The Use of a defined medium and mutants for establishing *Campylobacter* nutrition during colonisation

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DEDICATION

My deep dedication to my mother, without her encouragement and copious prayers, this study would have never been completed. Moreover, I wish to extend my dedication to my brothers, sisters and special thanks to devoted wife, my sweet lovely sons and daughter for patience and their interminable support without which this thesis could have never been finalized.
Abstract

A convenient defined medium was developed for use with *Campylobacter* species. Using this media *C. jejuni* NCTC 11168 was shown to be able to utilise mucin, L-serine, L-proline, L-glutamic acid, L-valine, L-glutamine, L-histidine, L-tyrosine, L-aspartate, L-asparagine and L-glycine as sole carbon sources. Amongst the sugars tested fucose, ribose and D-glucosamine hydrochloride supported growth. When different strains of *Campylobacter* were introduced into GMEM there were marked differences in growth. Out of six strains of *C. jejuni*, two grew well, whilst three out of five strains of *C. coli* grew well and two strains grew poorly in GMEM. Strains *C. coli* NCTC 11350, *C. coli* NCTC 11438 and *C. jejuni* NCTC 11951 failed to grow in GMEM alone, but when L-serine or L-glutamine were added NCTC 11438 and NCTC 11951 grew well, whilst the growth of NCTC 11951 was partially stimulated.

The addition of carbon sources during the survival of *C. jejuni* had different effects depending on their nature. When mucin was added 10 hours post survival, recovery was stimulated by 10-fold yet when metabolisable single carbon sources were added, survival was decreased by 6-logs suggesting that the provision of unbalanced nutrition induces substrate accelerated death.

This study also addressed potential mechanisms of mucin utilization in *C. jejuni*. Mutants deficient in SdaC (serine transporter) and in AspA (aspartate ammonia lyase) were shown to be deficient in serine and aspartate utilization respectively but both were still able to utilize mucin suggesting that the liberation of serine and aspartate from mucin is not important for its use as a carbon source. A plate based assay for mucinase/protease
activity, and an assay using *p*-nitrophenol derivatives of sugars, did not detect any mucinolytic or glycosidic activities in *C. jejuni*.

Mucin degradation is likely to generate short peptides and consequently, the mechanisms of peptide utilisation and transport were also investigated. Peptides containing either two or four aspartate residues supported some growth but this was less than when aspartate was provided alone. Peptides of four or seven residues containing serine supported the best growth. It would appear then that *C. jejuni* can utilize peptides containing aspartate and serine as carbon sources. Mutants deficient in Cj1580c-1584c, a putative ABC transport system for peptides, and Cj0653c, a putative aminopeptidase were still able to utilize peptides and neither system can be involved in the transport of the peptides assessed here. In addition a screen to isolate mutants unable to utilize peptides was developed using toxic peptide analogues. L-Ala-AEP (L-alanyl-L-aminoethylphosphonic) was show to be suitable for this purpose but not triornithine or bialophos as *C. jejuni* NCTC 11168 was naturally resistant to these. When different strains of *C. jejuni* and *C. coli* were assessed for L-Ala-AEP sensitivity, three out of three strains of *C. jejuni* tested were sensitive and three of four stains of *C. coli* tested were resistant. This most likely reflects the existence of different mechanisms of peptide uptake or degradation in strains of *Campylobacter*.

The new defined media also provided an opportunity to study the use of iron compounds and compatible solutes by *C. jejuni*. *C. jejuni* was shown to be able to acquire iron from DL- neorepinephrine, epinephrine, caffeic acid, rutin and quercetin but not catechin,
Abstract

although at high concentrations caffeic acid and rutin were growth inhibitory. Using GMEM it was found that at high osmolarities (0.15-0.3 M NaCl) the addition of the compatible solutes proline and betaine did not stimulate the growth of *C. jejuni* as is the case in other bacteria. In contrast, glycine betaine appeared to be growth inhibitory irrespective of the osmolality of the media.
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Chapter 1

General Introduction

1.1 History

Today, campylobacters are recognized globally as highly prevalent foodborne pathogens that cause diarrhoeal disease in humans (Gaynor et al., 2005). The name Campylobacter is derived from two greek roots: campylo (curved) and bacter (rod). Species names have other derivatives for example; jejuni was derived from the modern latin meaning of the jejunum, from which Campylobacter jejuni was originally isolated (Sebald and Veron, 1963).

The first report of Campylobacter enteritis may have been as long ago as 1880 Campylobacter-like organisms were thought to have been observed in the stools of infants with diarrhoea in 1880, by the German bacteriologist Escherich (Griffiths and Park, 1990). At the start of the 20th century, the microorganism, was originally identified as Vibrio fetus and was suspected to be responsible for spontaneous abortion in bovine and ovine hosts (McFadyean and Stockman, 1913). However, later it was found that this organism differed from Vibrio species in that it could not grow well under aerobic conditions, it had a spiral shape and did not ferment sugars. Consequently it was
renamed *Campylobacter* in order to differentiate it from *Vibrio* species (Catteau, 1995). Isolation of *Campylobacter* organisms was not successful until 1972 (Dekeyser *et al.*, 1972). In 1977 the development of selective media made isolation more straightforward and this began to reveal for the first time the true impact of these organisms (Skirrow, 1977).

Eighteen species of *Campylobacter* are recognised today, and of these only *C. jejuni* and *C. coli* cause significant human gastrointestinal disease (Vandamme *et al.*, 1995) (Table 1.1). *C. jejuni*, is recognised as the most frequent cause of human bacterial enteritis worldwide. In England and Wales about 47,030 cases were reported in 2007 (Health Protection Agency Centre, 2007). Also *C. coli* is a significant cause of foodborne disease. However, *C. coli* causes 5% of the gastrointestinal infection due to campylobacters whilst *C. jejuni* causes 95% (Chan, 2000).
Table 1.1: Species of *Campylobacter*, host and associated diseases

<table>
<thead>
<tr>
<th>Species of <em>Campylobacter</em></th>
<th>Host</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. jejuni</em></td>
<td>Man and sheep</td>
<td>Enteritis, systemic infection, perinatal disease, abortion</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>Man</td>
<td>Enteritis</td>
</tr>
<tr>
<td><em>C. fetus</em></td>
<td>Man, cattle and sheep</td>
<td>Meningitis, enteritis, systemic infection, perinatal disease, abortion</td>
</tr>
<tr>
<td><em>C. hyointestinalis</em></td>
<td>Man and pig</td>
<td>Proctitis, enteritis</td>
</tr>
<tr>
<td><em>C. sputorum</em></td>
<td>Man</td>
<td>Isolated from abscess pathogenicity</td>
</tr>
<tr>
<td><em>C. lari</em></td>
<td>Man</td>
<td>Enteritis</td>
</tr>
<tr>
<td><em>C. upsaliensis</em></td>
<td>Man, dog and cat</td>
<td>Enteritis</td>
</tr>
<tr>
<td><em>C. concisus</em></td>
<td>Man</td>
<td>Associated with periodontal disease</td>
</tr>
<tr>
<td><em>C. curvus</em></td>
<td>Man</td>
<td>Alveolar abscess</td>
</tr>
<tr>
<td><em>C. showae</em></td>
<td>Man</td>
<td>Isolated from the oral cavity pathogenicity unknown</td>
</tr>
<tr>
<td><em>C. gracilis</em></td>
<td>Man</td>
<td>Isolated from the oral cavity and visceral, head and neck infections</td>
</tr>
<tr>
<td><em>C. rectus</em></td>
<td>Man</td>
<td>Associated with periodontal disease</td>
</tr>
<tr>
<td><em>C. mucosalis</em></td>
<td>Pig</td>
<td>Necrotic enteritis, intestinal adenomatosis</td>
</tr>
<tr>
<td><em>C. hyoilei</em></td>
<td>Pig</td>
<td>Proliferative enteritis</td>
</tr>
<tr>
<td><em>C. helveticus</em></td>
<td>Man, cat and dog</td>
<td>Pathogenicity unknown</td>
</tr>
<tr>
<td><em>C. insuleenigrae</em></td>
<td>Seals, porpoises</td>
<td>None at present</td>
</tr>
<tr>
<td><em>C. lanienae</em></td>
<td>Cattle, pig and human</td>
<td>None at present</td>
</tr>
<tr>
<td><em>C. hominis</em></td>
<td>Human</td>
<td>Gastroenteritis in immunocompromised</td>
</tr>
</tbody>
</table>

Adapted from (Penner, 1988; Vandamme *et al*., 1995).
1.2 General biology and Culture

Campylobacters are described as Gram-negative rods, spiral-shaped, curved, generally 0.5-5.0 μm long and 0.2-0.8 μm wide (Figure 1.1). In older cultures rounded or coccoid forms often predominate. Campylobacters are non-spore forming and normally have a single polar, unsheathed flagellum present at one or both ends of the cell. This produces the characteristic rapid and darting corkscrew-like motility, a feature which aids their identification by phase contrast microscopy (Etoh et al., 1993; Vandamme et al., 1995).

Campylobacters are microaerobic, preferring an atmosphere of 3-15 % O2 and 3-5 % CO2. Some species are thermophilic, growing optimally at 42°C (Skirrow, 1977; Endtz et al., 1991). A general feature of campylobacters is that they are unable to ferment, or oxidise carbohydrates and instead utilise amino acids or tricarboxylic acid cycle intermediates, for energy metabolism (Griffiths and Park, 1990; Mohammed et al., 2004).

Campylobacter spp. are apparently fragile organisms that are not able to grow at temperatures below 30°C and are sensitive to the presence of atmospheric oxygen. Also unable to multiply outside the animal host and are highly susceptible to a number of environmental conditions compared with other food-borne bacterial pathogens (Table 1.2). In addition, the organism is sensitive to exposure to excessively high temperatures. The D-value for C. jejuni at 55°C is 1 min, and the z value is 5°C (Park et al., 1991). Therefore, sufficient heating may be useful to destroy Campylobacter in food. They are also sensitive to drying, surviving over a very restricted a_w range at room temperature,
although, Campylobacters can survive for several weeks at the appropriate humidity levels and temperatures of 4°C (Doyle and Roman, 1982b).

Figure 1.1: Scanning electron microscope image of *Campylobacter jejuni*, showing its curved and bipolar flagella (Altekruse *et al.*, 1999).
The optimum pH for growth is between pH 6.5 and 7.5 (Blaser et al., 1980a; Doyle and Roman, 1981). In addition, concentrations of 2% NaCl are also known to be inhibit growth (Doyle and Roman, 1982a).

Table 1.2: Environmental sensitivity of *C. jejuni* compared with other pathogenic bacterial

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Temp. °C range for growth</th>
<th>Minimum pH</th>
<th>Minimum aw</th>
<th>Typical D-value at 55°C</th>
<th>Oxygen requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>30-45</td>
<td>4.9</td>
<td>0.987</td>
<td>1.0</td>
<td>5-10%</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>0-45</td>
<td>4.4</td>
<td>0.92</td>
<td>4.5</td>
<td>Facultative</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>7-46</td>
<td>4.4</td>
<td>0.95</td>
<td>5.5</td>
<td>Facultative</td>
</tr>
<tr>
<td><em>Salmonella Typhimurium</em></td>
<td>5.2-46</td>
<td>3.8</td>
<td>0.93</td>
<td>4.7</td>
<td>Facultative</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>7-48</td>
<td>4</td>
<td>0.83</td>
<td>3</td>
<td>Facultative</td>
</tr>
</tbody>
</table>

*Data taken from International commission on microbiological specification for foods 1996 (Park, 2001).

Under stressful conditions campylobacters have been shown to transfer from a spiral to the coccoid form (Moran and Upton, 1987), a process that is usually accompanied by their conversion into a viable but nonculturable state (VNC) (Rollins and Colwell, 1986; Roszak and Colwell, 1987).
A wide range of media is available for the selective culture of campylobacters. All contain a nutrient base supplemented with reagents to protect cells from toxic derivatives of oxygen, and antibiotics to inhibit the growth of other bacteria. The first selective medium to be widely used was that described by (Skirrow, 1977). This contains blood agar base, lysed blood, trimethoprim, polymixin B and vancomycin. Although it is still used today, improvements have led to the development of several other media including Butzler’s medium Virion (Goossens et al., 1983), campy-BAP (Blaser et al., 1979) and Preston medium (Bolton and Robertson, 1982). All of these media contain blood which enhances the growth of fastidious microorganisms and can act as an oxygen quenching agent. Charcoal has been successfully used as an alternative to blood in selective media (Bolton and Coates, 1983), for example in modified CCDA medium, containing cefoperazone and amphoteracin B. However, due to the diversity of individual Campylobacter species in their growth requirements, and sensitivity to antimicrobial supplements, any one selective medium is unlikely to support the growth of all species. The addition of ferrous sulphate, sodium metabisulphite and sodium pyruvate (FBP) to the media has been shown to be beneficial in protecting against peroxide and superoxide (Griffiths and Park, 1990). Originally selective media were incubated at 42°C, allowing for the growth of C. jejuni and C. coli, but suppressing the growth of many other bacteria. However, it has become recognised that certain species and strains of Campylobacter, such as C. jejuni subsp. doylei, are unable to grow at this temperature and many other species have a lower optimum growth temperature. Therefore, in order to increase the isolation of the less common species an incubation temperature of 37°C is
now recommended (Griffiths and Park, 1990; Taylor et al., 1991; Bolton et al., 1992; Fernandez et al., 1997).

The minimal standards for identifying *Campylobacter* spp. have been suggested by various groups. After primary isolation colony morphology, Gram’s stain, motility and oxidase tests should be assessed (Butzler and Skirrow, 1979; Bolton, 2001). The hippurate hydrolysis test differentiates most *C. jejuni* strains from other *Campylobacter* spp. However, for organisms other than *C. jejuni* and *C. coli*, including atypical *C. jejuni* strains, additional biochemical tests are required. Several typing methods are available including serotyping (Penner and Hennessy, 1980; Lior et al., 1982; Frost et al., 1998), phage-typing (Frost et al., 1999), pulsed-field gel electrophoresis and genotyping (Wassenaar and Newell, 2000), and these can be used to characterise the strains further.

### 1.3 Clinical aspects

Campylobacteriosis is an acute gastroenteritis and can affect people of all ages but incidence appears to peak in young children of 1-4 years old and also in young adults between the ages of 15-24 years old (Varnam and Evans, 1991). The number of males acquiring infection is slightly higher than the number of females (Skirrow, 1994). The clinical manifestation varies from mild watery to severe bloody inflammatory diarrhoea and whether the infection is caused by *C. jejuni* or *C. coli* cannot be distinguished on the basis of these symptoms alone. Also the organisms generate significantly different symptoms depending upon whether infections occur in industrialized or developing nations. Patients in developing countries appear to have less severe symptoms than those
in developed countries (Nachamkin et al., 1992; Catteau, 1995; Altekruse et al., 1999; Park, 2001). This occurrence possibly reflects the differences in exposure rates and the development of protective immunity. The majority of patients with gastroenteritis in developing countries are young children. Acquired immunity in developing countries has shown to be age dependent (Lin et al., 1998; Prasad et al., 2001; Coker et al., 2002). Individuals in poorer countries tend to be infected with multiple *Campylobacter* serotypes and are frequently reinfected asymptotically with new serotypes. In developed countries illness generally begins with a fever associated with malaise and headaches followed with nausea and abdominal cramping persisting up to seven days and often resembling the symptoms of acute appendicitis with bloody diarrhoea. Although, the acute diarrhoea lasts only two to three days in some cases *C. jejuni* infections lead to bacteremia, septic arthritis, and other complications (Sorvillo et al., 1991). Furthermore, patients from industrialised countries are generally infected with only one *Campylobacter* serotype and are rarely reinfected (Sjogren et al., 1989). Usually *Campylobacter* enteritis is self-limiting (Blaser, 1997) but a significant if rare, sequelae for *C. jejuni* infection is Guillain-Barre Syndrome (GBS). This disease is a serious neurologic complication that may lead to paralysis. Although the trigger for this immune syndrome is not known, GBS is often preceded by *C. jejuni* infection (Mishu and Blaser, 1993; Walk, 1997), and it is estimated that one case of GBS occurs for every 1000 cases of campylobacteriosis (Altekruse et al., 1999). Infection with *Campylobacter* of Penner serotypes O: 19, O: 23 and O: 36 in particular, is associated with increased risk of the development of Guillain-Barre Syndrome (GBS) (Penner and Aspinall, 1997). Although,
*Campylobacter* does not commonly cause death, it has been suggested that about 500 persons with *Campylobacter* infections may die each year (NJDHSS, 2004).

### 1.4 Sources and transmission routes

Campylobacteriosis is mainly a food-borne infection in which foods of animal origin, particularly poultry, play an important role (Butzler, 2004). Food infected by cross-contamination may also present a significant source of infection since campylobacters have been found to contaminate several sites within domestic kitchens following the preparation of raw chicken (Cogan *et al.*, 1999). The incidence of infection tends to be sporadic and outbreaks are uncommon. More than 2 million cases occur yearly in the United States with almost all cases occurring sporadically (NJDHSS, 2004). Person-to-person transmission appears to be uncommon with *C. jejuni* (Robinson, 1981; Black *et al.*, 1988).

It has been suggested that most sporadic cases and small family outbreaks are associated with the consumption of undercooked poultry, or foods that have been cross-contaminated with raw poultry during storage or preparation (Skirrow, 1994). More recently Friedman *et al.*, (2000) have observed a strong association between *Campylobacter* infection and the handling and eating of raw poultry. Thus there appears to be a strong association of *C. jejuni* with poultry. Indeed, surveys have observed that 30-100% of poultry harbours *C. jejuni* as its normal gut flora (O'Sullivan *et al.*, 2000). Bashor *et al.*, (2004) found that there are several factors such as the washer system and chill tank that contribute to *Campylobacter* contamination of broiler carcasses. Other
routes of transmission that are most often associated with \textit{C. jejuni} infection are the consumption of unpasteurized milk which is regularly implicated as a vehicle (Tauxe, 1992; Shane, 2000). \textit{C. jejuni} may be present in milk from faecal contamination during milking or following an udder infection (Hutchinson \textit{et al.}, 1985; Orr \textit{et al.}, 1995). In addition untreated water in which \textit{C. jejuni} can survive for extended periods is well known source of infection (Tauxe, 1992; Shane, 2000). The first known outbreak of waterborne campylobacteriosis, affected around 2900 persons in Bennington, Vermont, and these individuals developed \textit{C. jejuni} enteritis after the town's water system became contaminated with water from an unfiltered source (Vogt \textit{et al.}, 1982). Since this time numerous waterborne outbreaks of \textit{C. jejuni} infection have been reported to the Centres for Disease Control (CDC), and between 1978 and 1986, 57 reported cases were all associated with consumption of untreated surface water or inadequately chlorinated water (Nachamkin \textit{et al.}, 1992). Furthermore, Richardson \textit{et al.}, (2007) identified that an outbreak of \textit{C. jejuni} infection in South Wales Valleys was due to contamination with surface water from nearby pasture land. In addition Furtado \textit{et al.}, (1998) found that some outbreaks were associated with exposure to swimming pool water. Other foods, including salad greens, fruit and fish have also been suggested as vehicles for \textit{Campylobacter} infection but generally are thought to represent a lower risk (I.I.D, 2000). In addition seafoods have been implicated in two outbreaks (Stern, 1992).

Wild birds are thought to be the reservoir of infection for commercial poultry flocks and cattle (Jones and Telford, 1991). Rosef (1981) isolated \textit{C. jejuni} from 24.8\% of a sample
of 129 wild birds which consisted of pigeons, seagulls and crows. In addition migratory birds that carry *C. jejuni* may also disperse the pathogen into lakes and rivers (Luechtelfeld *et al.*, 1980). Campylobacters are considered to be part of the normal intestinal flora of a wide range of domestic animals (Robinson, 1981) and close contact with infected animals is a significant cause of campylobacteriosis in children (Luechtelfeld *et al.*, 1981; Shane and Montrose, 1985; Kapperud *et al.*, 1992). Indeed, it is estimated that about 5% of human cases originate from contacts with pets, particularly dogs and cats (I.D.E, 2005). Other animals including dairy cows, which are infected by other reservoirs for *C. jejuni* including insects and feral animals also carry *C. jejuni* (Stern, 1992). Rodents will readily occupy farm environments and will be naturally attracted to spilled feedstuffs and wastes and since *C. jejuni* can be isolated from the faeces of rodents (Fernie and Park, 1977) these are also a possible source of farm contamination.

A striking aspect of *Campylobacter* incidence in temperate countries is the pronounced and consistent seasonal variation (Nylen *et al.*, 2002) with cases of human infection in the UK peaking in early June (Meldrum *et al.*, 2005). The increased rate of infection has been linked to increased temperatures (Louis *et al.*, 2005) which suggest that out door dining, flies or increased farming activity might be responsible.
Chapter 1

General Introduction

The fly as a vector for the spread of Campylobacteriosis has been considered Rosef (Rosef and Kapperud, 1983; Shane et al., 1985; Khalil et al., 1994; Hald et al., 2004; Nichols, 2005) and flies have been linked to an increase in campylobacter infections in England and Wales study (Nichols, 2005), between in May and June (Nichols, 2005). In Denmark, 8.2 % of flies that were isolated from outside of a broiler house carried Campylobacter spp. suggesting that flies act as vectors for their transmission (Hald et al., 2004). Insects when present in large numbers, may also represent a risk for the introduction of Campylobacter spp. into the chicken houses (Hansson et al., 2007).

1.5 Genetic content

The genus Campylobacter have small genomes which is thought to reflect their small size and limited nutritional properties (Trust et al., 1994). Before the advent of complete genome sequencing the genomes of C. jejuni and C. coli were estimated to be approximately 1.7 megabases (mbp) and were predicted to exist as single circular DNA molecules with a G+C content of about 30 mol %. Campylobacters also contain both plasmids and bacteriophages as extrachromosomal elements (Taylor, 1992). The first complete genome sequence for C. jejuni was published by Parkhill et al., (2000) but since then more genome sequences have been obtained for different strains of C. jejuni and other species (Fouts et al., 2005).

The genome sequence of C. jejuni NCTC11168 is 1,641,481 base pairs (bp) in length. At least 20 of 1,654 predicted coding sequences (CDS) probably represent pseudogenes and 94 % of the genome codes for proteins, making it the densest bacterial genome
sequenced at this time (Parkhill et al., 2000). This genome has recently been re-annotated and the total number of coding sequences reduced from 1654 to 1643 (Gundogdu et al., 2007). The genome contains no transposons, phage remnants, or insertion sequence elements and very few long repeat sequences. There appears to be little organization of genes into operons or clusters, however, there are a few exceptions, including genes for ribosomal RNA, lipopolysaccharide (LOS) synthesis, flagella modification and extracellular polysaccharide (EP) biosynthesis.

The initiation of transcription by sigma factors is a key step in the bacterial regulation of gene expression. Unlike Bacillus subtilis with 14 sigma factors or Escherichia coli with 7 sigma factors or Streptomyces coelicolor with 65, only three sigma factors (σ^{28}, σ^{54} and σ^{70}) were identified in the genome sequence of C. jejuni NCTC11168 (Wosten et al., 1998; Parkhill et al., 2000; Helmann, 2002). Also very few two-component transcriptional regulators have been predicted from the genome. This suggests, that compared to other bacteria, C. jejuni has a reduced capacity for sensing environmental parameters and adjusting gene expression accordingly. This feature is also consistent with its fastidious nature and environmental sensitivity (Park, 2002).

The Campylobacter species have different metabolic capabilities with some strains growing under both aerobic and anaerobic conditions (Carlone and Lascelles, 1982; Smibert, 1984). However, Fouts et al., (2005) found that certain species have very similar metabolic profiles based upon a comparison of the complete genome sequences of C. jejuni RM1221, C. coli RM2228, C. lari RM2100 and C. upsaliensis RM3195 with
the main variation being the presence of complete or partial tricarboxylic acid cycles. The tricarboxylic acid cycle in *C. jejuni* RM1221 appears to be intact and most likely serves a double role of generating biosynthetic compounds and providing intermediates that feed into electron transport. However, the other species appear to lack succinate dehydrogenase. In addition all four sequenced strains have pathways for the metabolism and biosynthesis of a number of amino acids and acetate, formate and lactate appear to be the main end products of carbon metabolism. For *C. jejuni* NCTC11168 the inability to utilize carbohydrates is thought to be a manifestation of the lack of phosphofructokinase which is necessary for glycolysis (Parkhill *et al.*, 2000; Velayudhan and Kelly, 2002). Nevertheless *C. jejuni* RM1221 has been reported to respire in the presence of fructose and mannose (Fouts *et al.*, 2005). This apparent conflict may reflect the conditions under which the substrates were tested or strain specific genome content. Variable patterns of substrate utilization have however been reported previously (Mohammed *et al.*, 2004).

*C. jejuni* is known to grow at the expense of amino acids *in vitro* and has been shown to utilize serine, aspartate, glutamate, and proline (Leach *et al.*, 1997). Consistent with these findings, the complete genome sequence of *C. jejuni* NCTC 11168 has revealed the presence of a number of homologues of amino acid-catabolizing enzymes (Parkhill *et al.*, 2000). L-serine is preferentially utilized compared to other amino acids (Leach *et al.*, 1997) and catabolism of L-serine to pyruvate and ammonia is carried out by a serine dehydratase (encoded by Cj1624c) (Velayudhan *et al.*, 2004). In the same study it was shown that an active SdaA is essential for colonization of the avian gut by *C. jejuni* and
this implies that catabolism of L-serine is crucially important for the growth of this bacterium in vivo.

1.6 Virulence

Virulence can be viewed as the extent to which a pathogen causes disease and a virulence factor may be defined as any property, expressed by the pathogen within the host, which contributes towards the disease process (Hensel and Holden, 1996). Specific inactivation of the gene encoding the putative virulence determinant should lead to loss or significant reduction in virulence (Falkow, 1988). Some properties may not contribute to the disease process directly, but may be involved in ensuring the survival and multiplication of the pathogen within the host such that the continued presence of the pathogen leads to disease. Such virulence factors include the adhesion to and invasion of the host cells. Others which may serve as general house keeping functions may also play a role in ensuring the continued survival of the pathogen within the host, and these factors too can be seen to contribute towards virulence (Mekalanos, 1992). Bacterial factors involved in the acquisition the iron come into this category. Iron acquisition mechanisms may be used by the pathogen outside the host but can also be used within the host to obtain iron complexed to iron binding proteins. Thus defining what constitutes a virulence factor is difficult, especially when it may vary between different strains of the same species. Such difficulties are particularly true for Campylobacter for which very few virulence determinants have been confirmed (Finlay and Falkow, 1997). The identification and characterization of different virulence factors will provide a better understanding of the
pathogenic mechanisms that are involved in *Campylobacter* infection (Grant *et al.*, 1993; Friis *et al.*, 2005).

Infection often begins with the translocation of bacteria, via motility to the site of infection. *Campylobacter* are motile bacteria with bipolar flagella and twisted cell morphology which also display both a directional tumbling and swimming motility and this behaviour has been linked to pathogenesis. A combination of the flagellum and cell shape is believed to give campylobacters an unusually high level of motility in viscous environments (Ferrero and Lee, 1988). The flagellum of *C. jejuni* is composed of a basal body, hook, and filament, and the flagellar filament is comprised of two proteins termed FlmA and FlkB. Both flagellin proteins are synthesized concomitantly (Nuijten *et al.*, 1991) but flaA is expressed at much greater levels than flaB, (Hendrixson *et al.*, 2001). Inactivation of flaA results in non-motile bacteria that express a truncated flagella and such mutants have been shown to be less adhesive to and invasive of epithelial cells *in vitro* (Wassenaar *et al.*, 1991), indicating that motility is an important pathogenic determinant. In addition, mutants with an inactivated flaA gene were shown to be attenuated in chicken caecal colonisation as compared to the wild-type. In comparison, a poorly motile variant with flagella composed solely of FlmA was found to colonize better than wild-type (Wassenaar *et al.*, 1993). The two flagella genes are independently transcribed as flaA is regulated by the sigma factor $\sigma^{28}$ (fliA), and flaB by $\sigma^{54}$ (rpoN). Accordingly, the expression of flaB can be influenced by changes in the level of nitrogen and pH (Guerry *et al.*, 1991; Jagannathan *et al.*, 2001). The cognate response regulator FlgR and its sensor kinase FlgS form a two component regulatory system which
regulates the fla regulon in *C. jejuni* (Wosten *et al.*, 2004). The interaction of a distinct environmental stimulus with FlgS, leads to the activation of kinase activity and as a result, FlgS autophosphorylates and subsequently transfers its phosphate to FlgR. FlgR stimulates the production of $\sigma^{28}$ and activates the transcription of genes necessary for the assembly of the hook-basal body filament complex (Carrillo *et al.*, 2004; Wosten *et al.*, 2004; Hendrixson, 2006). FlgQ and FlgP are also required for flagellar motility in *Campylobacter* species, although both are not components of the transcriptional regulatory cascades to activate $\sigma^{28}$- or $\sigma^{54}$-dependent expression of flagellar genes (Sommerlad and Hendrixson, 2007). Park *et al.*, (2000) found that flagellin synthesis can undergo phase variation due to the changeable gain and loss of a nucleotide, eventually affecting the production of the protein FlhA and thus flagellar motility in *C. coli*. The precise function of FlhA is unknown but it belongs to a family of proteins involved in the regulation or secretion of surface extracellular portions. However, *flhA* in *C. jejuni* probably does not use this phase-variable mechanism to control flagellar motility because it does not have the same homopolymeric nucleotide tract (Parkhill *et al.*, 2000). A similar mechanism, that is the loss or gain of a nucleotide in a homopolymeric adenine or thymine tract with *flgR* controls the expression of the FlgR response regulator (Hendrixson, 2006). Overall, the presence of the tight and complex regulation of flagella biosynthesis in *C. jejuni* can be explained by the requirement for highly motile bacteria for efficient colonisation of host (Wassenaar *et al.*, 1993; Wosten *et al.*, 2004) and also the fact that this apparatus is targeted by the immune system. An important and additional role for the flagella apparatus in campylobacters is that it appears to be required for the secretion of particular virulence factors, since mutants that
are affected in the ability to make the flagellin filament are unable to secrete the Cia (Konkel et al., 2004). More recently, FspA, a small acidic protein that induces apoptosis in host cells has also been shown to be secreted through the flagella filament (Poly et al., 2007).

Several reports suggest that chemotaxis is an important C. jejuni virulence determinant. Campylobacters have mechanisms to detect chemical gradients and linked motility functions that enable the cell to move up or down the gradient. The importance of chemotaxis has been demonstrated by testing chemically mutagenized, non-chemotactic mutants in animal models (Takata et al., 1992). Yao et al., (1997) studied the in vitro and in vivo role of chemotaxis using a set of defined C. jejuni mutants. A C. jejuni cheY null mutant was generated, and found to display a nonchemotactic but motile phenotype. This C. jejuni mutant exhibited an increase in adherence and invasion of INT 407 cells when compared to the wild-type isolate, but was unable to colonize mice or cause symptoms in infected ferrets. A possible explanation for these findings is that the motility of a cheY mutant is altered such that the organism makes longer runs, resulting in increased host cell contacts that promote irreversible cell adherence and invasion. In vivo, the increase in the lengths of the runs in vivo, without chemotaxis providing appropriate directionality towards mucus, could lead to the organism's expulsion from the host by fluid flow and peristaltic activity. Thus, the chemotactic response of C. jejuni appears important in directing the organism to specific sites in the host's intestinal tract. Chemoattractants that may mediate this process include mucin, and components of mucin, such as fucose and certain organic acids (Hugdahl et al., 1988). However, the signal receptors and
mechanisms involved in the resulting signal transduction system that influences motility are still poorly understood.

Flagellins from various strains of *C. jejuni* and *C. coli* have been shown to be glycosylated (Doig *et al.*, 1996; Thibault *et al.*, 2001). The glycosyl modifications are surface-exposed and highly immunogenic in the flagellar filament (Power *et al.*, 1994). It is now known that several cell surface features other than the flagellins, including Peb3 and CgpA are glycosylated (Linton *et al.*, 2002). There are two glycosylation pathways in this organisms. That for the *N*-linked protein glycosylation system modifies dozens of proteins (Karlyshev *et al.*, 2005), and the *O*-linked glycosylation system only modifies flagellar subunits (Szymanski *et al.*, 2003). A number of genes involved in *O*-glycosylation of *Campylobacter* flagellin have been described (Guerry *et al.*, 1996; Szymanski *et al.*, 1999). All genes identified to be involved in glycosylation of campylobacter flagellins map near the two flagellin structural genes, *flaA* and *flaB* and have variable regions, one region approximately from base positions 700 to 1,450 and a short variable region (SVR) from base positions 450 to 600 that probably corresponds to surface-exposed parts of the proteins in the *C. jejuni* chromosome (Meinersmann *et al.*, 1997; Dorrell *et al.*, 2001; Pearson *et al.*, 2004; Fouts *et al.*, 2005).

Mass spectrometry and Nuclear magnetic resonance spectroscopy (NMR) experiments have shown that the major modification sugars on flagellin from *C. jejuni* are based on 5,7-diacetamido-3,5,7,9-tetradecoxy-L-glycero-L-manno-nonulosonic aci or pseudaminic acid (Pse) and related derivatives (Thibault *et al.*, 2001; McNally *et al.*, 2005).
2006). The other general protein glycosylation pathway (Pgl) that is responsible for the
N-linked addition of a heptasaccharide containing N-acetylgalactosamine, glucose, and
2,4-diacetamido-2,4,6-trideoxy-α-D-glucopyranose (2,4-diacetamido-Bac) to at least 30
different proteins (Wacker et al., 2002; Young et al., 2002). Changes in flagellin
glycosylation affect Campylobacter autoagglutination and influence adherence and
invasion of intestinal epithelial cells (Guerry et al., 2006). Therefore, the glycans on the
surface of the flagella filament are likely to play an important role in virulence (Guerry et
al., 2006).

Another class of cell surface molecules that contain sugar modifications is the capsular
devsaccharide. The capsule of C. jejuni was identified after a large gene cluster
encoding various genes with similarities to type II/III capsular polysaccharide-related
genes from other bacteria were found in the course of the C. jejuni NCTC 11168 genome
sequencing project (Parkhill et al., 2000). The capsule has since been characterized and
found to be the major component of the Penner serotyping scheme (Karlyshev et al.,
2000).

1.7 Toxins
Since C. jejuni was first identified as an agent of human disease many groups have
reported the presence of various toxins, including cytotoxins, enterotoxins or both
(Wassenaar, 1997). For many years there was conflicting evidence as to whether
Campylobacters produce a cholera-like enterotoxin (CLT) and other cytotoxins (Ruiz-
Palacios et al., 1983; McCardell et al., 1984; Johnson and Lior, 1986). However, the
only toxin substantiated by the publication of the complete genome sequence is cytolethal distending toxin (CDT) (Johnson and Lior, 1988; Lara-Tejero and Galan, 2000; Mooney et al., 2001; Mooney et al., 2003). This toxin appears to be widespread in campylobacters since it was reported that 41% of 718 isolates examined produced CDT (Mooney et al., 2003). In susceptible cells, toxin activity is evident from cell distension characterized by both elongation and swelling to more than 4 times normal size (Johnson and Lior, 1988). Enlargement of nuclei is also common in the distended cells. Finally, CDT-treated cells crumble or die. Cell lines found to be susceptible to CDT include Caco-2, Vero, REF52, HeLa, CHO, COS-1, HEp-2, and INT 407 cells (Johnson and Lior, 1988; Whitehouse et al., 1998; Pickett and Whitehouse, 1999; Lara-Tejero and Galan, 2000). The sensitivity of different cell lines to CDT is variable, which may be due to differences in their surface receptors (Pickett and Whitehouse, 1999). The toxin is encoded by three overlapping genes, *cdtA*, *cdtB* and *cdtC*. The DNAase activity of the *cdtB* product has been shown to be responsible for the observed morphological changes with CdtA and CdtC being needed for delivery (Lara-Tejero and Galan, 2000).

Whilst its activity has been established, the role of Cdt during infection remains less clear. Patients with disease symptoms elicited antibody against the toxin but in asymptptomatically colonised chickens antibodies are not seen which suggests a role during human infection only (Pickett *et al*., 1996; Eyigor *et al*., 1999). In addition, Cdt-negative mutants are able to colonise poultry in the same manner as wild type strains (Biswas *et al*., 2006). The isolation of naturally occurring Cdt-negative strains, albeit
rarely, from symptomatic cases of human infection would suggest however, that it is not
an essential virulence factor (Abuoun et al., 2005).

1.8 Adhesion

Adhesins are surface-exposed molecules that facilitate a pathogen’s attachment to host
cell receptor molecules (Konkel et al., 2001). Adhesion prevents pathogens from being
washed away by bodily fluids and may also be the first step towards host cell invasion
(Zhang and Normark, 1996), although not all C. jejuni strains that have been shown to
adhere will necessarily invade (Konkel and Joens, 1989). As a prelude to adhesion to
enterocytes campylobacters must first colonise mucus. Lee et al., (1986a) observed that
C. jejuni specifically associated with the intestinal mucus-blanket and mucus-filled
crypts of BALB/c mice. This association involved highly motile organisms with no
apparent adhesion to epithelial cells of the gut mucosa. However, mucus association was
only studied over the course of several days due to experimental difficulties in
maintaining depletion of normal surface-associated bacteria. In addition, the relevance
of the model is debatable because C. jejuni-infected mice do not develop disease. These
investigators hypothesized that the lack of pathology in the mouse model was a result of
the host cells lacking the appropriate receptors for bacterial products. The interaction of
C. jejuni with mucin was later investigated by Szymanski et al.,(1995) using non-
polarized Caco-2 cells and carboxymethylcellulose. These investigators found that the
increased viscosity imparted by the carboxymethylcellulose resulted in longer runs by C.
jejuni. The longer runs were proposed to result in an increase in the frequency of
contacts between *C. jejuni* and host cells, thus leading to increases in host cell adherence and invasion. Based on these observations, the association of *C. jejuni* with the mucus in the crypts, it also being viscous, was proposed to be important for cell invasion.

A number of potential adhesins have been characterised in *C. jejuni* (Konkel *et al.*, 2005). One of the first potential adhesins to be considered was the flagella (McSweegan and Walker, 1986). Flagellated but non-motile cells of *C. jejuni* attach to epithelial cells more efficiently than non-flagellated variants suggesting that adhesion is associated with the flagellum (Newell *et al.*, 1985a). In a later study, however, which examined non-motile flagellated mutants and non-motile non-flagellated mutant it was found that aflagellated and flagellated, nonmotile *C. jejuni* adhered to cultured human epithelial cells as readily as the wild-type parent suggesting that the flagella play no role in (Grant *et al.*, 1993). However, on the basis that aflagellated mutants were deficient in the ability to enter the epithelial cells the authors did suggest a role for flagella or flagellin in internalization. An additional study, which demonstrated that antibodies directed against flagella had no effect on the adherence of *C. jejuni* to INT 407 cells (Konkel and Cieplak, 1992) further suggests that flagella or flagellin are not essential for adherence. This is further supported by the findings of Wassenaar *et al.*, (1991) who found that the addition of purified flagellin did not competitively inhibit the binding of *C. jejuni* to cultured cells. Two antigenic proteins, PEBI (Pei *et al.*, 1998) and PEB3 that are commonly recognized by convalescent sera from patients with sporadic *C. jejuni* diarrhoea have been suggested to be adhesins based on the fact that mutants in these proteins adhere to epithelial cells less efficiently and colonise animal models less well (Pei *et al.*, 1998). In
addition, to its potential role in adhesion PEB3, has been shown to be homologous with the periplasmic-binding proteins associated with ABC transporters and thus also plays a key role in the utilization of aspartate and glutamate (Leon-Kempis Mdel et al., 2006) which may be important in vivo for the utilization of carbon sources for this pathogen during infection. Carbohydrate modification of bacterial glycoproteins has been shown to affect adhesion and in this context in is interesting to note that PEB3 can be modified via glycosylation (Young et al., 2002; Linton et al., 2002).

Extracellular matrix proteins may be important in campylobacter adhesion. A fibronectin (Fn) binding protein called CadF was identified by Konkel et al., (1997) using a binding assay and immobilized extracellular matrix proteins, and since CadF mutant C. jejuni have significantly reduced binding to fibronectin (Fn) this has been suggested to be the major adhesion factor for fibronectin (Konkel et al., 1997). Monteville et al., (2003) confirmed the specificity of C. jejuni binding to Fn, through CadF by using antibodies reactive against fibronectin and CadF. In addition, a mutant deficient in cadF was unable to colonise the caeca of chicks suggesting a role for cadF in colonization (Ziprin et al., 1999). The domain within CadF that mediates binding to fibronectin has recently been shown to reside in four amino acids (AA134-137). Genes encoding the jlpA lipoprotein and PEB1 adhesins have also been mutated, and the phenotypes examined. A C. jejuni jlpA knockout is reduced 19% in adherence to HEp-2 cells (Jin et al., 2001).

The binding of C. jejuni to INT 407 cells has also been suggested to be mediated by LPS. This suggestion was based on observations that radioactively labeled LPS bound to INT
407 cells and that pretreatment of INT 407 cells with LPS inhibited adhesion (McSweegan and Walker, 1986). However, a mutation in the galE gene, involved in LPS synthesis, had no effect on the level of chicken colonization compared to wild-type even though there was a reduction in adhesion to, and invasion of, INT 407 cells (Fry et al., 2000). Other suggested adhesins include JlpA (jejuni lipoprotein A), a surface exposed lipoprotein (Jin et al., 2001) and the major outer membrane protein (MOMP, also called (OmpE) (Moser and Schroder, 1997; Moser et al., 1997).

The pilli of Salmonella Enteritidis and Escherichia coli have been shown to have a role in mediating adherence (Doig et al., 1996; Dibb-Fuller et al., 1999; La Ragione et al., 2000) and if such structures exist in C. jejuni they may also have a role in adhesion. The production of pilli has been observed in C. jejuni following exposure to the bile salt deoxycholate, and whilst in vitro assays demonstrated that pilli played no role in promoting the organism’s adherence or invasion of epithelial cells, in vivo studies revealed that the a C. jejuni putative peptidase pspA mutant exhibited reduced pathology in ferrets when compared to animals infected with the C. jejuni wild-type isolate (Doig et al., 1996). A more recent study however has suggested that the bile induced pilli are an artifact since they were also observed when bile was added to culture media in the absence of Campylobacter cells (Gaynor et al., 2001).

1.9 Intracellular survival and Oxidative stress

The ability of C. jejuni to bind to host tissues and to survive, and replicate in mammalian cells has been extensively examined by using tissue culture models (Newell et al., 1985b;
Konkel and Joens, 1989; Konkel et al., 1990; Pei et al., 1998; Rivera-Amill et al., 2001; Friis et al., 2005). Clinical C. jejuni isolates have been shown to possess varying degrees of invasiveness (Newell et al., 1985b). Also Fauchere et al., (1986) found that C. jejuni strains isolated from those with fever and diarrhoea had greater binding to epithelial cells than strains isolated from individuals without fever and diarrhoea. However, a subsequent study has been unable to support the correlation between invasiveness and clinical manifestation (Tay et al., 1996).

C. jejuni invasion has been reported to be optimal when mammalian cells are inoculated at a low multiplicity of infection (MOIs) (Hu and Kopecko, 1999; Mooney et al., 2003). However, Biswas et al., (2000) reported that the maximal number of internalized bacteria occurs at higher MOIs.

Expression of Campylobacter invasion antigen B gene (ciaB) is thought to required for invasion of INT 407 cells but not for adhesion (Konkel et al., 1999). CiaB is identified in the cytoplasm of intestinal epithelial cells and exported across the inner and outer membranes without a periplasmic intermediate (Rivera-Amill et al., 2001). Once internalized, C. jejuni organisms can survive for extended periods of time within epithelial cells and ultimately induce a cytotoxic response (Konkel et al., 1992). In vitro C. jejuni is able to survival and replicate inside macrophages and human monocytic cell vacuoles and induces apoptotic death via cytolethal distending toxin (Hickey et al., 2005). Human monocytes when infected with Campylobacter produce a range of proinflammatory cytokines and chemokines, including IL-8, IL-1β, IL-6 and tumor
necrosis factor alpha. This role in infection may be crucial to the development of
inflammatory disease (Jones et al., 2003; Siegesmund et al., 2004). Bacterial infection
of epithelial cells stimulates the production of chemokines that are involved in the
attraction of leukocytes (Jung et al., 1995; Bakhiet et al., 2004). Therefore, the
infections interact with leukocytes to stimulate the immune response. It has been
suggested that the stimulation of leukocytes is significant and could contribute to the
pathology of disease (Jones et al., 2003).

Recently, the cytokine responses produced during the interaction of Campylobacter
infection with the avian host have been studied (Smith et al., 2005). The cytoskeleton of
the eukaryotic cell is mostly comprised of tubulin and actin, and these components have
been shown to be involved in pathogen invasion, in both cellular and subcellular
movements, and in the determination of cell shape (Finlay and Falkow, 1997). C. jejuni
strain 81-176 has been shown to associate with filamentous structures comprised of
polymerised microtubule proteins, during the first stage of the invasion process of
INT407 cells (Hu and Kopecko, 1999). Recent studies suggest that some strains depend
on both microfilaments and microtubules for invasion and that tyrosine protein kinase
linked pathways play a role in transducing extracellular signals that induce invasion
(Biswas et al., 2000; Biswas et al., 2004).

1.9.1 Oxidative stress

Generally, in their natural environment, whether in the gastrointestinal tracts or in the
external environment during transmission, it is probable that C. jejuni cells are faced with
growth-limiting or potentially lethal conditions, such as oxidative stresses (Baillon et al., 1999). However, campylobacters are generally considered to be micro-aerophilic implying an inherent sensitivity towards oxygen and its reduction products (Park, 2002). It is, however, possible to grow campylobacters in the presence of air under certain conditions (Jones et al., 2003). Under stressful conditions campylobacters have been shown to transfer a process that is from a spiral to the coccoid form (Moran and Upton, 1987), usually accompanied by their conversion into a viable but nonculturable (VNC) state (Rollins and Colwell, 1986; Roszak and Colwell, 1987). The VNC state relates to a bacterial cell that remains infectious but that can no longer be cultured by conventional means, and was first proposed by Colwell following a study on the survival of Salmonella in aquatic systems (Roszak et al., 1984). Bacteria in this state may retain metabolic activity but are unable, under the prevailing environmental conditions, of undergoing the cellular division required for growth. Furthermore, conversion from the VNC form has been shown to be reversible with the advent of improved environmental conditions (McKay, 1992). Clearly, the presence of a VNC form of Campylobacter would have significance for the detection and epidemiology of this pathogen. The existence of this state is controversial however and there has been continuous debate as to whether a VNC form for Campylobacter actually exists. Superoxide dismutase rather than the catalase is essential for the survival during air exposure (Purdy et al., 1999; Stead and Park, 2000).

In C. jejuni, the expression of genes associated with oxidative-stress resistance is required to protect the organism from reactive oxygen species produced during normal
microaerobic metabolism, during exposure to air, and when in contact with the host defence system. The main superoxide defence mechanism is superoxidase dismutase (SodB), which requires iron as a co-factor. The product of sodB, SodB catalyses the conversion of oxygen radicals to hydrogen peroxide and dioxygen. This enzyme was identified as important for C. jejuni survival intracellularly in INT407 (Pesci et al., 1994), and in air and in model food systems (Purdy et al., 1999). In addition, SodB is important for the efficient colonisation of chicks (Purdy et al., 1999; Baillon et al., 1999).

The other main oxidative stress mechanism in C. jejuni is involved in peroxide defence. Alkyl hydroperoxides are catalase (KatA) and alkyl hydroperoxide reductase (AhpC) are both repressed the peroxide stress regulator (PerR) (van Vliet et al., 1999). Catalase converts hydrogen peroxide, produced by SOD, to oxygen and water. Even though katA mutants are sensitive to hydrogen peroxide, they are not altered in their ability for aerobic growth or colonisation of chicks (Purdy et al., 1999). However, katA is important for intramacrophage, but not interaepithelial, survival (Day et al., 2000). Alkyl hydroperoxide reduces alkyl hydroperoxides to alcohols, and as a result becomes oxidized itself. The oxidized AhpC protein may be reduced by the ferredoxin protein, Fdx, in order to recycle the reduced AhpC (van Vliet et al., 2001). C. jejuni encounters elevated levels of nitrosative stress during infection, since nitric oxide (NO) synthesis is markedly increased in patients with infective gastroenteritis (Forte et al., 1999; Enocksson et al., 2004). During infection, both macrophages and enterocytes generate bactericidal concentrations of NO through the action of inducible NO synthases
(Salzman et al., 1998; Witthoft et al., 1998; Weinberg, 1999; Alderton et al., 2001) and resistance to this radical is likely to be important during intracellular survival. Bacteria possess a number of activities that detoxify NO and its redox products (Poole, 2005). In C. jejuni, the single domain globin, Cgb performs a major NO scavenging and detoxification function in this pathogen (Elvers et al., 2004). NssR (Cj0466), a member of the Crp-Fnr superfamilly, has been identified as the major positive regulatory factor that controls nitrosative stress-responsive expression of this gene and an NssR-dependent nitrosative stress responsive regulon that consists of a least four genes (Elvers et al., 2005). This regulon clearly plays an important role in protecting C. jejuni from the toxic effects of NO but its role in vivo is yet to be established.

1.10 Colonization

Colonisation is important for Campylobacter for two reasons, it serves to maintain the organism in its natural animal reservoir and it also is a prelude to human infection. There are a number of important factors that assist enteric bacteria such as Campylobacter to colonize the gastrointestinal mucus, such as chemotaxis, flagella, pilli and adhesins (Hugdahl et al., 1988; Wallis, 1994; Ziprin et al., 1999). These have been discussed in detail previously. Chemotactic studies on C. jejuni by Hugdahl et al.,(1988) have shown that the glycoprotein mucin, a principle constituent of mucus, is chemoattractant to the organism, and several individual components of mucin are also chemoattractant including L-fucose, a terminal sugar on the oligosaccharide units of mucin. In addition, physicochemical factors such as temperature, pH, water activity,
oxidation reduction, and presence or absence of certain substrates have been found to influence gastrointestinal colonization (Sinell, 1980).

Takata et al., (1992) found that *C. jejuni* mutants unable to respond to known chemoattractants, such as L-fucose, were unable to colonise the mouse intestinal tract. The reduced virulence of this chemotactic mutant was likely due to an inability to move toward the chemoattractant mucin (Hugdahl et al., 1988). These results suggest that chemotaxis is crucial to colonisation. Furthermore, a recent publication identified two genes, *cetA* (containing an N-terminal transmembrane region that would localize the protein to the bacterial inner membrane) and *cetB* (a cytoplasmic protein containing PAS domain), which may enable campylobacters to migrate to new environments and maintain maximal electron transport and ATP generation for energy chemotaxis in *C. jejuni* (Hendrixson et al., 2001). Both *cetA* and *cetB* mutants of *C. jejuni* were deficient in chemotaxis toward fumarate and pyruvate. Fumarate is the preferred terminal electron acceptor even in the presence of oxygen and pyruvate is rapidly metabolised by *C. jejuni* in the presence of oxygen as a terminal electron acceptor (Hendrixson et al., 2001). Consequently, *C. jejuni* is able to sense and respond to its intracellular energy status by using a bipartite system consisting of CetA and CetB in a manner analogous to Aer-mediated energy taxis in *E. coli* (Bibikov et al., 1997; Nichols and Harwood, 2000).
1.10.1 Animal host

*Campylobacter* species are considered to be a commensal in the intestines of certain domestic animals with avian hosts, especially chicken, regarded as the primary reservoir for *C. jejuni*. In general the organisms mainly colonize the mucus layer in the crypts of the intestinal epithelium of the caeca, large intestine and cloaca (Beery *et al.*, 1988). Once a strain has colonised, *C. jejuni* can rapidly reach high numbers $10^9$ CFU in the cecal contents in experimentally challenged birds (Wassenaar *et al.*, 1993). However, this level may be lower in naturally colonised birds (Saleha *et al.*, 1996). The large intestine and caeca are specialised environments and *C. jejuni* has evolved a number of strategies that allow it to exploit these ecological niches. For example, the optimal growth temperature of the organisms (42°C) mirrors that of the avian gut and this differs considerably from that encountered in the mammalian gut (37°C). The RacRS regulon, previously characterised as a two component regulatory system, is required for the differential expression of proteins at 37°C and 42°C (Brás *et al.*, 1999) and appears to be important in colonisation as inactivation of *racR* reduced the ability of the organism to colonize the alimentary tract in chickens (Brás *et al.*, 1999). The finding that danJ mutants are severely retarded in growth at 46°C and also unable to colonise chickens suggests that the heat shock response plays an important role in both thermotolerance and colonisation (Konkel *et al.*, 1998). Furthermore, when HspR and HrcA, which are both regulators of the heat shock response, are inactivated colonisation of the chicken gut is reduced reaffirming the importance of this response during colonisation (Stintzi *et al.*, 2005).
Other aspects of their physiology suggest that campylobacters are adapted to occupy the mucous layer of the gastrointestinal tract. Firstly, their microaerophilic nature is probably a reflection of the concentration of oxygen encountered in this niche. When C. jejuni is located in the mucous layer of the chicken gut, it is likely to be surrounded by a microaerobic atmosphere optimal for its growth. If however, the organism becomes detached, it may enter the lumen of the colon which is strictly anaerobic. The discovery of genes in the genome sequence encoding a number of terminal reductases which could potentially allow the use of a wide range of alternative electron acceptors to oxygen such as fumarate and nitrate led to speculation that under specific conditions C. jejuni could grow in the absence of oxygen. However, C. jejuni has not been shown to grow under strictly anaerobic conditions with any of the above electron acceptors but since these compounds increased growth in environments in which the rate of oxygen transfer was severely limited (Sellars et al., 2002), these alternative respiratory pathways may contribute to energy conservation in non-growing cells under oxygen-limited conditions in vivo.

When the intestinal lifestyle of C. jejuni has been studied using whole-genome microarrays, in both the rabbit ileal loop model (Stintzi et al., 2005) and in the chicken ceacum (Woodall et al., 2005), a central theme apparent in the pattern of up-regulated genes is a response to limited oxygen conditions since many of the up-regulated genes identified in the microarray screens are homologues of genes from other bacteria that are activated on exposure to low oxygen tensions or during anaerobiosis. However, it would also seem that the cells are also exposed to oxidative stress during colonisation as
inactivation of the sodB gene in campylobacters not only reduces intracellular survival in INT-407 cells (Pesci et al., 1994), but markedly also reduces colonization of 1-day-old chicks (Purdy et al., 1999).

_Campylobacter_ must also be able to survive under the stresses that are imposed by the upper gastrointestinal tract such as the pH of the stomach and exposure to bile salts. Campylobacters encounter bile during colonisation and resistance to this appears to be an important during colonisation. Bile contains a group of detergent-like bile salts that are secreted into the small intestine for digestion of fats, but since they are amphipathic molecules which also destroy the lipid bilayers of cell membranes they also exhibit potent bacterial activity. As a consequence of this, resistance to bile salts is an important characteristic for bacteria which colonise the intestinal tract. In poultry the concentration of bile salts range from 0.01% in the ceacum to 0.7% in the jejunum (Lin et al., 2003) and thus _C. jejuni_ must be able to tolerate these levels of this bactericidal agent during colonisation. The only known mechanism of bile salt resistance in this organism is the multidrug efflux pump CmeABC (Lin et al., 2002; Pumbwe and Piddock, 2002; Luo et al., 2003; Akiba et al., 2006) which consists of an outer membrane channel (CmeC), an inner membrane drug transporter (CmeB) and a periplasmic protein (CmeA) (Lin et al., 2002; Pumbwe and Piddock, 2002; Luo et al., 2003). Together these components form a membrane channel for the extrusion of broad spectrum of antimicrobials, including bile (Lin et al., 2002). A CmeB-deficient mutant dose not grow in bile containing media and fails to colonise the chicken intestinal tract (Lin et al., 2003) demonstrating the importance of this transporter in bile resistance and growth _in vivo_. The components of
the transporter are encoded by a three gene operon (Lin et al., 2002; Pumbwe and Piddock, 2002; Luo et al., 2003) and the expression of this operon is under the control of the repressor CmeR which is located immediately upstream of cmeABC (Lin et al., 2005). The inhibition of CmeABC activity by efflux pump inhibitors increases the sensitivity of C. jejuni to bile salts and reduces colonisation of chickens (Lin and Martinez, 2006) and consequently this transporter is thus a potential target for therapeutic intervention to reduce colonisation of animals and humans (Quinn et al., 2007).

The ability of C. jejuni to become established in the gastrointestinal tract of chickens is also believed to involve binding of the bacterium to its surface cells. The outer membrane protein CadF, which facilitates the binding of Campylobacters to fibronectin, is thought to be responsible for this since a deficient mutant is incapable of colonizing the caecum of newly hatched chicks (Ziprin et al., 1999).

1.10.2 Human host

Many factors that influence colonisation of the poultry gut such as resistance to bile, nutrition and iron acquisition, will be relevant to colonisation of the human gut and only likely differences will be considered here.

Directly after ingestion in the human host C. jejuni is exposed to the low pH of stomach and its ability to survive in this environment is essential for infection. Compared to other food-borne pathogens C. jejuni appears to be unusually sensitive to low pH (Cuk et al., 1987). However, several Campylobacter strains appear to have increased acid
resistance compared with commonly used strains such as 81116 and NCTC 11351 (Murphy et al., 2003). One such acid-tolerant strain, C1120, exhibits an adaptive tolerance response to acid, which requires de novo protein synthesis, and which can be induced by sublethal exposure to acid and aerobic conditions (Murphy et al., 2003).

The organism may be also exposed to other stresses within the stomach. The reduction of food-borne nitrate to nitrite in the mouth has been associated with the oral microbial flora on the posterior tongue (Duncan et al., 1995; Li et al., 1997). Once nitrite enters the stomach, it encounters hydrochloric acid and these results in the chemical generation of nitric oxide, which is bactericidal. In the stomach, this mechanism is thought to provide a powerful defence against gut pathogens (Duncan et al., 1995; Dykhuizen et al., 1996). As considered previously (Section 1.9 Oxidative stress) C. jejuni possesses a protein Cgb, which is a homologue of the single domain globins and this protein plays a role in defence of C. jejuni against nitrosative stress (Elvers et al., 2004) and thus may play a protective role against this stress.

After it passes from the stomach C. jejuni will be exposed to bile salts, low oxygen, hyperosmolarity, and iron limitation in the small intestine. The response to low oxygen and iron are discussed elsewhere (Section 1.9 and 1.9.1). The chme that passes from the stomach into the intestine is generally not considered to be hyperosmotic, but as its macromolecular components are digested the osmolarity may increase dramatically and an osmolarity of 700 milliosmoles per kg H₂O (Hallback et al., 1978) has been reported although it is not known how C. jejuni adapts to increases in osmolarity. However,
Campylobacters are much less tolerant of osmotic stress than other bacterial foodborne pathogens (Doyle and Roman, 1982a) and this may be a reflection of their limited capacity to respond to osmotic stress and the lack of adaptive mechanisms for this (Park, 2002).

1.11 Iron acquisition

Iron is an essential nutrient for all living organisms and the capability to obtain iron from the host contributes to bacterial pathogenesis throughout the infection process (van Vliet and Ketley, 2001). In order to colonize the intestine, Campylobacters must be able to compete with the resident flora for this vital element. Campylobacters have not been shown to produce siderophores, but they are able to use exogenous siderophores (Field et al., 1986). Numerous iron-acquisition systems have been identified in Campylobacters, including a haemin uptake system (ChuA) (Ridley et al., 2006), an enterochelin-transport system (Richardson and Park, 1995; Palyada et al., 2004) and an Fe (II)-transport system (FeoB) (van Vliet et al., 2002; Raphael and Joens, 2003). Additionally, a putative ferrichrome uptake system has been suggested (Galindo et al., 2001) and two other uptake systems, Cj0178-Cj0181 and p19 might be present (van Vliet et al., 2002). Enterochelin uptake and Fe$^{2+}$ transport may play an important role in colonisation as Palyada et al., (2004) have shown that ferric enterobactin receptor (cfrA) mutants, defective in enterobactin-mediated iron acquisition, were unable to colonize the gastrointestinal tract and also a feoB mutant was significantly affected in both its ability to transport Fe$^{2+}$ and to colonise the chick caecum (Naikare et al., 2006). Iron storage
systems are also used by microorganisms to allow growth in low-iron environments. In addition, such storage systems help to protect the bacterium against iron overload, which may result in iron-catalysed oxidative damage to cellular components. The iron storage protein ferritin is produced by \textit{C. jejuni} (Wai \textit{et al.}, 1995) and a \textit{C. jejuni} mutant in the gene encoding ferritin, \textit{cft} (Wai \textit{et al.}, 1996), was found to grow poorly in iron-deficient media and was sensitive to oxidative stress. Thus, production of ferritin may facilitate the colonization of the host by \textit{C. jejuni} and may also help protect the bacterium in conditions of high oxygen levels.

As is the situation in many other bacteria, in \textit{C. jejuni} the regulation of iron uptake systems is influenced by iron availability and is mediated by the ferric uptake regulator. Fur binds to specific DNA sequence (Fur boxes) overlapping Fur-regulated promotors when the intracellular Fe\textsuperscript{2+} concentration is high enough to allow the formation of a complex consisting of a Fur dimer and Fe\textsuperscript{2+} (van Vliet \textit{et al.}, 1998). The iron-transport systems for haemin (ChuABCD), ferric iron (FeoAB) and enterochelin (CeuBCDE), as well as the putative iron-transport genes \textit{p19}, \textit{Cj1658}, \textit{Cj0177}, \textit{Cj0178} and \textit{cfirA}, all appear to be regulated by Fur as they are all expressed at elevated levels in the wild-type strain under iron limitation and in the \textit{fur} mutant in iron-rich conditions (Holmes \textit{et al.}, 2005).

Norepinephrine NE is one of the major neurotransmitters of the nervous system. The dense innervation of the mesenteric organs is mostly responsible for the production of a large amount of the body's NE (Lundgren, 2000). A large proportion of NE released by
these neurones escapes breakdown at the site of release and spills over into the
circulation where it is spread throughout the mesenteric tissues (Aneman et al., 1996).
Trauma-induced destruction of noradrenergic neurones and subsequent release of NE are
known to stimulate the growth of the intestinal flora in the gut of mice (Lyte and Bailey,
1997). Also exposure of bacteria to this hormone increases the adherence of the E.coli
O157 to tissue in vivo (Vlisidou et al., 2004). Recently, a study by Cogan et al.,(2007)
reported that exposure of Campylobacter to NE led to greater numbers of motile and
more virulent cells.

1.12 Nutrition in the gut

Some nutrients essential for Campylobacter growth may be poorly available or
unavailable to the bacteria within the gut lumen. However, these nutrients, such as iron,
may be available from host tissues. Bacteria can produce toxins or invade cells to access
such host materials by damaging the integrity of the host intestinal mucosa during
infection. Such strategies inevitably have pathogenic consequences for the host.

The discovery of genes encoding a number of terminal reductases which could
potentially allow the use of a wide range of alternative electron acceptors to oxygen such
as fumarate, nitrate and nitrite raised the possibility that under specific conditions C.
jejuni could grow in the absence of oxygen. However, none of these compounds support
growth under strictly anaerobic conditions and these pathways have thus been suggested
to play a role in energy conservation under oxygen limited conditions (Sellars et al.,
2002). Previous studies found that succinate dehydrogenase Sdh in E. coli can function
as an effective fumarate reductase in vitro (Pershad et al., 1999; Leger et al., 2001). Also it has been shown to be capable of supporting anaerobic growth (Maklashina et al., 1998). However, Sdh is highly activated in *C. jejuni* in vivo to maintain the oxidative function of the citric-acid cycle under the low oxygen tensions found in the gut (Woodall et al., 2005).

The carbon sources that *C. jejuni* can utilize in the gut are unknown, although the most likely source of carbon and nitrogen in vivo is via deamination of amino acids, given that *C. jejuni* is asaccharolytic (Smibert, 1984; Velayudhan and Kelly, 2002). Amino acids are likely to be deaminated to a small number of intermediates that can directly feed into the central metabolism, including pyruvate, oxaloacetate, and 2-oxoglutarate. Also, ammonia has been generated from deamination, which can be used as a nitrogen source. However, there is not much information on which amino acids might be important in vivo. *C. jejuni* can grow in minimal media by using aspartate, serine, proline, and glutamate as carbon sources (Leach et al., 1997). Therefore, serine catabolism appears to be especially important in the normal physiology of *C. jejuni* (Velayudhan and Kelly, 2002). Serine can be deaminated by SdaA catalysis, producing pyruvate and ammonia, both of which can be readily assimilated (Grabowski et al., 1993). A diversity of evidence indicates *sdaA* in *C. jejuni*, is the structural gene for L-serine deaminase. Mutants with null mutations in the *sdaA* gene are unable to synthesize it (Velayudhan et al., 2004). In addition, Velayudhan et al., (2004) found that serine transport in *C. jejuni* is carried out by at least two systems, a lower-affinity L-serine-specific transporter
encoded by \textit{sdaC} and a higher-affinity transporter that was revealed by analysis of the residual transport kinetics of the \textit{sdaC} mutant.

In the intestinal lumen, ingested feed moves distally while digestion and absorption occur, and at the same time, a wide range of microbial species are supported. Absorptive processes occur at the brush border, which encompasses extensions of the epithelial surface. This surface is covered with a mucous gel secreted by epithelial goblet cells that acts as a protective barrier against harmful intraluminal components (Gork \textit{et al.}, 1999). Furthermore it is the initial barrier with which enteric drugs and nutrients must interact and diffuse through, in order to be absorbed and to enter the circulatory system and their target end organs (Bansil and Turner, 2006). Since \textit{C. jejuni} is thought to occupy this environment during colonisation of its animal host and during the infection of humans it is possible that this pathogen can acquire nutrition from mucus. The primary components of mucous are mucins, which are high-molecular-weight proteins characterised by a variable number of tandem repeat peptide sequences rich in amino acids which carry large numbers of $O$-linked oligosaccharide chains (Schroten \textit{et al.}, 1992; de Repentigny \textit{et al.}, 2000). Mucus is a complex gel of glycoproteins and glycolipids (Allen, 1984). In addition, the mucous layer contains 95\% of water, salts and other important elements such as phospholipids which may have an effect on bacterial translocation (Usui \textit{et al.}, 1999). 19 mucin genes (designated MUC) have been recognized cloned and sequenced in the human, and animal. However, only three MUC (MUC1, MUC2 and MUC5B) genes have been totally sequenced due to the large size of
the central tandem repeats, which are difficult to accurately assemble (Perez-Vilar and Hill, 1999).

The sugar substituents of mucus include N-acetylgalactosamine, N-acetylglucosamine, fucose, galactose, sialic acids, and smaller amounts of glucuronate and galacturonate. The majority of enteric bacteria require a fermentable carbohydrate for growth, and fermentation is assumed to be the mode of metabolism used by most species (Salyers and Leedle, 1983). Most carbohydrate in the colon is in the form of mucosal polysaccharides, which are degraded by a few anaerobes that dominate the intestinal biota. The monosaccharides released from mucin and other mucosal glycoproteins support the growth of many intestinal bacteria such as \textit{E. coli}, which does not make polysaccharide-degrading enzymes (Corfield \textit{et al.}, 1992) and also presumably \textit{C. jejuni}. Based upon whole genome expression profiling (Chang \textit{et al.}, 2004) have suggested that there are seven sugars including gluconate, \textit{N}-acetylglucosamine, \textit{N}-acetylneuraminic acid, glucuronate, mannose, fucose and ribose used by \textit{E. coli} MG1655 as nutrients during colonization of the mouse intestine. (Chang \textit{et al.}, 2004) identified genes in \textit{E. coli} that were induced by mucus, a condition designed to mimic the mammalian intestine. Most of the induced genes corresponded to catabolic pathways for compounds found in mucus. Based on this gluconate appears to be the major carbon source used by \textit{E. coli}, at least in the mouse intestine. As a commensal organism of animals and a pathogen in humans, \textit{Campylobacter} colonizes the intestinal mucus layer in the crypts of the intestinal epithelium. Mucus which is continually secreted by intestinal epithelial cells is rich in nutrients and can also readily support the growth of \textit{Campylobacter} (Beery
et al., 1988), but little is known as to whether C. jejuni can degrade mucins or which components of them it can utilise.

Aims of this study

Given the lack of information on the factors which influence Campylobacter colonisation as this applies to nutrition, iron acquisition and osmoregulation, the aims of this study were as follows:

- To develop a convenient defined media to investigate carbon nutrition in C. jejuni.
- Identify major carbon sources likely to be used by C. jejuni in the intestine, particularly those derived from mucin.
- Characterize some of the catabolic pathways for these nutrients.
- Use the defined media to investigate osmoregulation and novel sources of iron.
- Study the effect of these carbon sources Campylobacter survival.
Chapter 2

Materials and Methods

2.1 Culture and strains

2.1.1 Bacterial strains

The strains of bacteria used in this study are given in Table 2.1.

2.1.2 Microbiological media and media supplements

Media and media supplements were supplied by Oxoid (Unipath) unless otherwise stated. Media were prepared with deionised water from a Nanopure Ultrapure water system (Barnstead Int.) and sterilized by autoclaving at 121 °C for 15 minutes. To select for antibiotic resistance, antibiotic supplements were added aseptically to cooled, sterile media at the following concentrations; ampicillin 50-100 μg ml⁻¹, tetracycline 10 μg ml⁻¹, chloramphenicol 10 μg ml⁻¹ and kanamycin 50 μg ml⁻¹. All antibiotics were supplied by Sigma Chemical Company.

2.1.3 Growth and maintenance of cultures

Frozen stocks were kept at -80 °C in Microbank vials. Campylobacter spp. were routinely grown on Mueller-Hinton agar or in Mueller-Hinton broth. Campylobacter species were incubated at 37 °C under microaerobic conditions generated in a gas jar.
using a gas generating kit (Campy Gen Oxoid). Unless stated otherwise *Escherichia coli* strains were grown aerobically at 37°C on Luria- Bertani media (Oxoid). For survival studies of *C. jejuni* under aerobic conditions Maximum Recovery Diluent MRD (Oxoid Ltd, Basingstoke, Hampshire, UK) was used to suspend cells and Blood-Muller Hinton-agar (5% sheep blood) (BMH agar) was used to improve recovery of damaged cells.

### 2.1.4 Assessment of various defined media suitable for *C. jejuni* and the use of carbon sources

Liquid cultures (50 ml) of *C. jejuni* were grown microaerobically with gentle agitation in different media supplemented with FeSO₄ (50 μM) and HEPES (25mM) when necessary in tissue culture flasks (200 ml) with vents (Nunc, Roskilde, Denmark). Two defined media were investigated: Minimum Essential Medium α medium 1X powder (MEM, Invitrogen) and Glasgow Minimum Essential Medium liquid (GMEM, Invitrogen). GMEM was used as supplied whilst MEM was made up according to the manufactures instructions. Briefly, 5.04g MEM α was dissolved in 500 ml sterile deionised water and stirred until completely dissolved. It was then filter sterilised (0.2 μm Nalgene MF75 SFCA sterilization unit, Nalgene Int) and transferred into sterile bottles. Carbon sources (Table 2.2) were added as stated in the results section.
<table>
<thead>
<tr>
<th>Bacteria strain</th>
<th>Genotype or relevant characteristics</th>
<th>Source and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter jejuni</td>
<td>NCTC 11168 (genomic strain)</td>
<td>'NCTC</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>NCTC 11951 (type strain)</td>
<td>NCTC</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>NCTC 11351 (type strain)</td>
<td>NCTC</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>NCTC 11847 (type strain)</td>
<td>NCTC</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>NCTC 11322 (type strain)</td>
<td>NCTC</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>NCTC 11828 (type strain)</td>
<td>NCTC</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>Δ Cj 1580c -1584c:: KanR</td>
<td>Professor D. Maskell (University of Cambridge)</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>Cj 0653c::Tn, CmR</td>
<td>Professor D. Maskell (University of Cambridge)</td>
</tr>
<tr>
<td>CjNAS1</td>
<td>11168 sdaC:: KanR</td>
<td>This study</td>
</tr>
<tr>
<td>CjHAR8</td>
<td>11168 aspA:: Asp CmR</td>
<td>H. Talbot, University of Surrey</td>
</tr>
<tr>
<td>Campylobacter coli</td>
<td>UA585</td>
<td>Parental strain (Wang and Taylor, 1990)</td>
</tr>
<tr>
<td>Campylobacter coli</td>
<td>NCTC 11350</td>
<td>NCTC</td>
</tr>
<tr>
<td>Campylobacter coli</td>
<td>NCTC11366</td>
<td>NCTC</td>
</tr>
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<td>Campylobacter coli</td>
<td>NCTC 12110</td>
<td>NCTC</td>
</tr>
<tr>
<td>Campylobacter coli</td>
<td>NCTC 11438</td>
<td>NCTC</td>
</tr>
<tr>
<td>Campylobacter coli</td>
<td>NCTC 11437</td>
<td>NCTC</td>
</tr>
<tr>
<td>E. coli</td>
<td>F 80dlacZ ΔM15</td>
<td>Life Technologies</td>
</tr>
</tbody>
</table>

'NCTC, National collection of type cultures (Colindale); Kan' kanamycin resistance; Ap' ampicillin resistance; Cm' chloramphenicol resistance.
Table 2.2: Amino acid sugars used in the study

<table>
<thead>
<tr>
<th>No</th>
<th>Compounds</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L- Alanine</td>
<td>Sigma</td>
</tr>
<tr>
<td>2</td>
<td>L- Arginine</td>
<td>Sigma</td>
</tr>
<tr>
<td>3</td>
<td>L- Aspartic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>4</td>
<td>L-Asparagine</td>
<td>Sigma</td>
</tr>
<tr>
<td>5</td>
<td>L- Cysteine</td>
<td>Sigma</td>
</tr>
<tr>
<td>6</td>
<td>L- Glutamine</td>
<td>Sigma</td>
</tr>
<tr>
<td>7</td>
<td>L- Glutamic acid potassium salt</td>
<td>Sigma</td>
</tr>
<tr>
<td>8</td>
<td>L- Glycine</td>
<td>Sigma</td>
</tr>
<tr>
<td>9</td>
<td>L-Histidine</td>
<td>Sigma</td>
</tr>
<tr>
<td>10</td>
<td>L-Methionine</td>
<td>Sigma</td>
</tr>
<tr>
<td>11</td>
<td>L-Lysine</td>
<td>Sigma</td>
</tr>
<tr>
<td>12</td>
<td>L-Proline</td>
<td>Sigma</td>
</tr>
<tr>
<td>13</td>
<td>L-Threonine</td>
<td>Sigma</td>
</tr>
<tr>
<td>14</td>
<td>L-Valine</td>
<td>Sigma</td>
</tr>
<tr>
<td>15</td>
<td>L- Serine</td>
<td>Sigma</td>
</tr>
<tr>
<td>16</td>
<td>L-Phenylalanine</td>
<td>Sigma</td>
</tr>
<tr>
<td>17</td>
<td>Sodium pyruvate</td>
<td>Sigma</td>
</tr>
<tr>
<td>18</td>
<td>Fucose</td>
<td>Sigma</td>
</tr>
<tr>
<td>19</td>
<td>Ribose</td>
<td>Sigma</td>
</tr>
<tr>
<td>20</td>
<td>Mannose</td>
<td>Sigma</td>
</tr>
<tr>
<td>21</td>
<td>N-acetyl-D-glucosamine</td>
<td>Sigma</td>
</tr>
<tr>
<td>22</td>
<td>D-glucosamine hydrochloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>23</td>
<td>N-Acetyleneuraminic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>24</td>
<td>D-glucuronic acid sodium</td>
<td>Sigma</td>
</tr>
<tr>
<td>25</td>
<td>Keto-D-gluconic acid potassium</td>
<td>Sigma</td>
</tr>
<tr>
<td>26</td>
<td>L-Tyrosine sodium salt</td>
<td>Sigma</td>
</tr>
<tr>
<td>27</td>
<td>Gluconate sodium</td>
<td>Sigma</td>
</tr>
<tr>
<td>28</td>
<td>Glutamic acid solution</td>
<td>Sigma</td>
</tr>
<tr>
<td>29</td>
<td>Mucin (Porcine stomach type III)</td>
<td>Sigma</td>
</tr>
<tr>
<td>30</td>
<td>Asp</td>
<td>Sigma</td>
</tr>
<tr>
<td>31</td>
<td>Asp-Asp</td>
<td>Sigma</td>
</tr>
<tr>
<td>32</td>
<td>Asp-Asp-Asp-Asp</td>
<td>Sigma</td>
</tr>
<tr>
<td>33</td>
<td>Arg-Gly-Asp-Ser</td>
<td>Sigma</td>
</tr>
<tr>
<td>34</td>
<td>Arg-Gly-Asp-Ser-Pro-Ala-Ser</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
The media were inoculated with 1ml of harvested cultures of *C. jejuni* NCTC11168 (prepared by adding defined media to an overnight confluent MHA plate and adjusting OD$_{600}$ nm to 0.6). Samples were collected at regular intervals from the cultures to monitor growth by assessing optical density of the culture at 600nm (OD$_{600}$) using Pharmacia Biotech Ultra spec 200 UV visible spectrophotometer.

The utility of modified GMEM was also assessed for a number of different *Campylobacter* strains including *Campylobacter coli* NCTC12110, *Campylobacter coli* NCTC11437, *Campylobacter coli* NCTC11350, *Campylobacter coli* NCTC11366, *Campylobacter coli* NCTC11438, *Campylobacter jejuni* NCTC11828, *Campylobacter jejuni* NCTC11322, *Campylobacter jejuni* NCTC11351, *Campylobacter jejuni* NCTC11951, *Campylobacter jejuni* NCTC11847 and *Campylobacter jejuni* NCTC11168. Here 50 ml of GMEM, supplemented with FeSO$_4$ (50 pM), HEPES (25 mM) and sodium pyruvate (20 mM), in a tissue flask, was inoculated at zero time with one strain and this was grown at 37 °C in microaerophilic conditions with shaking. This experiment was preformed three times on three independent occasions and similar reproducible result obtained.

### 2.1.5 Plating counting

Ten-fold, serial dilution series were prepared from 1 ml of culture, using recovery diluent (MRD; 0.85% NaCl, 0.1% bacteriological peptone; Oxoid Ltd, Basingstoke, Hampshire, UK). Cultures were made in duplicate or triplicate and 0.1 ml aliquots of the cultures
were spread plated onto MH agar. The plates were incubated at 37°C under microaerobic conditions for 48 hours. Colonies were counted using a Quebec colony counter (Bibby Serrilin Ltd, Stone, UK) and counts expressed as CFU ml⁻¹.

### 2.2 Growth and survival studies

#### 2.2.1 The use of iron limited GMEM and other media to assess the use of compounds as iron sources by *C. jejuni*

The ability of *C. jejuni* to use various sources of iron was initially assessed using a disc diffusion assay, prepared using GMEM supplemented with sodium pyruvate (20mM) and HEPES buffer (25mM). Double strength GMEM, sterilized by filtering through a 0.22 μm filter was added to an equal volume of 3 % (v/v) molten and sterile technical agar. When the media had cooled to 50 °C, 1 ml of *C. jejuni* grown overnight in 100 ml of MH broth at 37°C was added to 500 ml of molten media. The culture was gently mixed in and the media poured into Petri dishes. After the plates had dried filter paper discs (Oxoid, Basingstoke; 6 mm in diameter) were placed onto the agar and of 10 μl of stock solutions added. Compounds added were 1mg/ml haemin and 100 mM FeSO₄, 0.750 μM caffeic acid, 0.750 μM DL-neorepinephrine, 0.750 μM rutin, 0.750 μM quercetin, 0.750 μM catechin and 0.750 μM epinephrine. Haemin 1mg/ml was dissolved in 0.1 M NaOH. DL-neorepinephrine was diluted in phosphate buffered saline (PBS). Rutin, epinephrine and quercetin were dissolved in saline, whilst catechin, FeSO₄ and caffeic acid were dissolved in water. All compounds were supplied by Sigma and stocks were prepared
fresh and used within 30 min of preparation. All solutions containing iron compounds were filter sterilized using a 0.2 μm syringe-tip filter (Gelman Acrodisc, Ann Arbor, MI). Growth around the disks was recorded after 24 h of incubation at 37 °C under microaerophilic conditions.

Liquid iron limited GMEM was prepared by supplementing GMEM with sodium pyruvate (20mM) and HEPES buffer (25mM) but with no addition of FeSO₄. The ability of C. jejuni to use compounds as sources of iron was assessed by adding haemin (100 μM), FeSO₄ (100 μM), caffeic acid (0.750 μM), DL- neorepinephrine (0.750 μM), rutin (0.750 μM), quercetin (0.750 μM), catechin (0.750 μM) and epinephrine (0.750 μM) to tissue flasks. Cultures were grown at 37 °C in microaerobic conditions with shaking and the growth assessed by measuring OD₆₀₀nm at selected time points. This experiment was preformed three times on three independent occasions and similar reproducible result obtained.

2.2.2 The use of GMEM to assess the use of compatible solutes by C. jejuni during growth at elevated osmolarity

Unlike Mueller Hinton broth, GMEM lacks known compatible solutes and it was thus used to assess the ability of C. jejuni to use various compatible solutes. MHB and GMEM were prepared with and without 0.1, 0.15, 0.2 and 0.3 M NaCl. When necessary, proline (1 mM) and glycine betaine (1 mM and 0.1 mM) were added to GMEM. Tissue culture flasks (250 ml capacity with vents) were filled with GMEM (50 ml) and
inoculated with 1 ml of a stationary phase culture of \textit{C. jejuni} from MH-broth that had been centrifuged (25000g for 10 minutes) in 2 x 50 ml centrifuge tubes (Bibby sterilin Ltd, Stone, UK), washed once with GMEM, and finally resuspended in the same volume GMEM. Growth was measured by assessing OD$_{600nm}$ at regular intervals when cultures were grown at 37°C in microaerobic conditions with shaking.

2.2.3 The survival of \textit{C. jejuni} in aerobic conditions at 25°C in response to the addition of various carbon sources

\textit{C. jejuni} cells were grown overnight in 50 ml of Muller Hinton broth for 24 hours at 37°C. The cell suspension was diluted 100-fold using MHB and then one ml aliquots were transferred to tissue flasks of 250 ml capacity with vents containing 100 ml MRD. The flasks were incubated at 25 °C in aerobic conditions with shaking at 150 rpm. Survival was monitored by plate counting at regular intervals on Blood MHA. Serine (20 mM), sodium pyruvate (20 mM), threonine (20 mM) or aspartate (20 mM) were added at various time intervals 5, 10 or 20 hours.

2.3 Peptide transport and utilization methods

2.3.1 Plate based protease and mucinase assays

To investigate the production of putative enzymes involved in mucin degradation, plates for assaying protease (Sizemore and Stevenson, 1970) and mucinase (Oliver \textit{et al.},
1986) activity were prepared. Solid GMEM agar was prepared as described in section 2.2.1. Protease detection plates comprised of this agar base with caesin (2% skimmed milk). Nonfat powdered milk 2 gram was dissolved in 50 ml of distilled water (w/v) and autoclaved at 121°C for 25 minutes and then was added to 50 ml of GMEM agar. For mucinase assay plates (2% mucin), 2 gram of mucin was dissolved in 50 ml of distilled water (w/v) and autoclaved at 121°C for 25 minutes then was added to 50 ml of GMEM. The plates were patched with single colonies of different C. jejuni and C. coli strains and cells grown for five days under microaerobic conditions at 37 °C. After this time the plates were assessed for clearing zones surrounding the growth, indicative of proteolysis of casein or mucin.

2.3.2 Preparation of crude extracts and assays for glycosidic enzymes

The method used was an adaptation of that of Tzortzis et al., (2003). Crude extracts for the assessment of glycosidic enzyme activity were obtained by growing cultures of C. jejuni in 50 ml of GMEM containing 1 % mucin for 48 hours. Aliquots (20 ml) were taken and centrifuged at 30,000g for 20 min and the cell pellets washed twice with 0.2 M potassium phosphate buffer (pH 6.8). The cell pellets were resuspended in the 2 ml of the same buffer. 100 μl of the cell suspension was mixed with 300 μl of the substrate solution containing each of the following substrates (5 mM) in 0.2 M potassium phosphate buffer (pH 6.8). The substrates used were p-nitrophenol-alpha-L-fucopyranoside, p-nitrophenol-alpha-D-glucopyranoside, p-nitrophenol-beta-D-glucopyranoside, p-nitrophenol-alpha-D-galactopyranoside or p-nitrophenol-beta-D-
galactopyranoside. The reactants were mixed and then the mixture was incubated at 37°C for two minutes. A solution of 2 ml disodium tetraborate (0.2 M) was the added to the mixture to stop the reaction and the release of nitrophenol was measured from the absorbances read at 400 nm in a spectrophotometer (Tzortzis et al., 2003).

2.3.3 The growth of C. jejuni in the presence various peptides

To establish whether various peptides could be used as sole carbon sources by C. jejuni, GMEM was prepared as described previously (2.1.4) but with no added carbon source. When necessary the media was supplimented with L-serine, sodium pyruvate, or aspartate (5mM), Asp-Asp (5mM), Asp-Asp-Asp-Asp (5mM), Arg-Gly-Asp-Ser (5mM) and Arg-Gly-Asp-Ser-Pro-Ala-Ser (5mM). Tissue culture flasks containing 20 ml of liquid media were inoculated with 0.2 ml of C. jejuni grown overnight in 50 ml of MH broth at 37°C and cultures were grown at 37 °C in microaerobic conditions with shaking. Growth was assessed by measuring OD\textsubscript{600nm} either during growth or at the end of the experiment (5 days) depending on the nature of the experiment. The strains of C. jejuni used in this part of the study were, NCTC 11168 (wild type) Cj 0653c::Tn, cmR (putative peptidase mutant), and ΔCj1580c -1584c:: kanR (putative peptide transporter mutant).
2.3.4 Assessment of the effect of toxic amino acid derivatives on the growth of *C. jejuni*

This method was adapted from the study of Abouhamad *et al.*, (1991). As a broad screen to assess the sensitivity of *C. jejuni*, and mutants deficient in putative peptide transport systems and peptidase activity, to various toxic peptide analogues a disc diffusion as was used. This was carried out using either MH-agar or GMEM-agar. GMEM supplemented with FeSO$_4$ (100µM), sodium pyruvate (40mM) and HEPES buffer (50mM) was added to an equal volume of 3 % (v/v) molten and sterile technical agar after it had cooled to 50°C then immediately inoculated 1 ml of *C. jejuni* NCTC 11168, Δ Cj 1580c -1584c:: Kan$^R$ or Cj 0653c::Tn, Cm$^R$ (provided by Professor Maskell, University of Cambridge) grown overnight in 100 ml of MH broth at 37°C. The culture was gently mixed in and the molten media poured into Petri dishes. Assessment was performed by placing filter paper discs (Oxoid, Basingstoke; 6 mm in diameter) onto the agar and adding 10 µl of each of the following compounds, 100 µM or 50 µM of Bialophos (Duchefa Biochemie), L-alanyl-L-aminoethylphosphonic (Ala-AEP) (Fluka) or Triornithine (Bachem). Plates were incubated at 37°C for 3 days. All the compounds used were made up as stock solutions in sterile distilled water and filter sterilized using a 0.22 µm syringe filter and immediately used.
2.3.5 The growth of *Campylobacter* strains in liquid media containing L-Ala-AEP

GMEM containing sodium pyruvate (20 mM), FeSO₄ (50 μM), HEPES (25 mM) was prepared with and without 200 μM L-Ala AEP. One ml of overnight cultures of various *C. jejuni* strains and *C. coli* strains, grown in MH broth under microaerophilic conditions for 24 hours, were inoculated at zero time into the media (50 ml) and incubated at 37°C with shaking and the growth was assessed by measuring OD₆₀₀ at selected time points. This experiment was performed three times on three independent occasions and similar reproducible results obtained.

2.3.6 The determination of the concentrations of L-Ala AEP that would be selective on GMEM-agar

An aliquot (0.1 ml) of an overnight culture of *C. jejuni* 11168 NCTC was placed into 5 ml of MHB then incubated in microaerobic conditions at 37 °C for 6 hours. Cell suspensions were diluted 10⁻¹, 10⁻² and 10⁻³ in MRD medium and were plated onto GMEM-agar containing sodium pyruvate (20 mM), FeSO₄ (50 μM), HEPES (25 mM) and with different concentrations of L-Ala-AEP (200 μM, 250 μM or 300 μM). L-Ala-AEP was made up as a stock solution in sterile distilled water and filter sterilized using a 0.22 μm syringe filter. The plates were incubated at 37°C for 4 days with daily inspection.
2.4 DNA methodologies

2.4.1 Plasmids

The plasmids used and generated during the study are listed in Table 2.3.

Table 2.3: Plasmids

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Genotype or relevant characteristics</th>
<th>Source and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD-TOPO</td>
<td>Cloning vector; ( \text{Ap}^R )</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pJMK30</td>
<td>Cloning vector; source of the ( \text{Kan}^R ) resistance cassette, ( \text{Ap}^R ), ( \text{Kan}^R )</td>
<td>Baillon et al., (1999)</td>
</tr>
<tr>
<td>pNAS1</td>
<td>pBAD containing ( \text{sdaC} ), ( \text{Ap}^R )</td>
<td>This study, University of Surrey</td>
</tr>
<tr>
<td>pNAS2</td>
<td>pBAD containing ( \text{sdaC} ), with a ( \text{Kan}^R ) cassette cloned into unique ( \text{BglII} ) site of ( \text{sdaC} )</td>
<td>This study, University of Surrey</td>
</tr>
</tbody>
</table>

2.4.2 Bacterial plasmid DNA isolation

The purpose of this technique was to provide purified plasmid DNA from \textit{E.coli}. The DNA was subsequently used for various procedures, including cloning. The technique was undertaken using a QIAprep Spin Miniprep Kit purchased from QIAGen. A 5 ml culture of the plasmid containing strain was produced by inoculating a 5 ml LB broth, containing an appropriate antibiotic, with a single colony of the desired bacterial strain using a sterilised wooden toothpick under aseptic conditions. The inoculum was then
incubated overnight at 37°C whilst being shaken at 220 rpm. Following incubation the plasmid DNA was then isolated following the manufacturer’s instructions. Briefly, 5 ml cultures were centrifuged to produce a cell pellet. The supernatant was discarded and the cells resuspended, via pipetting, in the manufacturer’s Buffer P1 (250μl). The cells were then lysed by applying Buffer P2 (250μl), and inverting the solution 6 times. The lysis reaction was allowed to occur for no longer than 2 minutes and was stopped by the application of manufacturer’s Buffer N3 (350μl) and by inverting a further 6 times. This solution was then spun in a bench top centrifuge for 10 minutes at 13000 rpm. Following centrifugation the supernatant was removed from the cell debris and applied to a manufacturer’s spin column. The column was then spun for 1 minute at 13000 rpm and the flow-through discarded. The column was then washed using manufacturer’s Buffers PB (500μl) and PE (750μl). These were applied consecutively to the column, with a 1 minute centrifugation step after each. A final spin for 2 minutes was used to remove any remaining PE Buffer. Finally, plasmid DNA was eluted into an Eppendorf tube, via centrifugation, by washing the column with 30μl of manufacturer’s Buffer EB. When required, plasmid DNA in EB Buffer was stored frozen at -20°C.

2.4.3 Purification of DNA fragments from reaction mixtures

The QIAGen PCR purification kit was used for this procedure. The purpose was to purify DNA fragments from contaminating molecules used in reactions such as PCR and restriction digestion. 5 volumes of manufacturer’s PB buffer were mixed with the
reaction volume, and the resulting sample was applied to a manufacturer's spin column. This was centrifuged at 13000 rpm for 1 minute. DNA bound to the column was then washed with 750 µl manufacturer's buffer PE via centrifugation at 13000 RPM for 1 minute. After the column run-through had been discarded, a further 1 minute of centrifugation was applied to discard any residual buffer. DNA was eluted into a sterile tube in 30 µl of manufacturer's EB buffer by centrifugation.

2.4.4 Purification of DNA fragments by gel-extraction

The purpose of this procedure was to purify DNA fragments of a particular size identified via agarose gel electrophoresis. The procedure was undertaken using a QIAquick Gel Extraction Kit supplied by QIAGen. The agarose gel electrophoresis technique served to separate fragments of different sizes and provided a means to visualise them via ethidium bromide staining. The desired fragment could then be selected. Once identified the fragment was extracted from the gel slab using a scalpel and transferred to a 1.5 ml Eppendorf tube. The gel plug was weighed, and its weight converted into volume. The conversion from weight to volume was made according to the supplier's instruction, and assumed 1 g of gel was equivalent to 1 ml. Three times the gel volume of manufacturer's QG buffer was added to the Eppendorf containing the gel plug and this was incubated at 50°C in a water bath for 10 minutes. Approximately every three minutes within the incubation period the tube was removed and its contents briefly agitated using a vortex. This helped the gel to melt more quickly. Once liquid,
the tube contents were applied to the column, although where fragments were shorter than 0.5 or longer than 4 Kb, 1 gel volume of isopropanol was added prior to this. QIAgen advised this enabled binding of such fragments to the matrix of the column. It was not necessary for fragments within this range. Following DNA binding, the column was washed with 500 µl of manufacturer’s QG buffer by applying this volume to the column and the column then being spun for 1 minute at 13000 rpm. The column was then washed with 0.75 ml manufacturer’s PE buffer. A final spin was used to remove any residual PE buffer before the purified DNA fragments were eluted off the column, and into a clean Eppendorf tube using 30 µl manufacturer’s EB buffer.

2.4.5 Agarose gel electrophoresis for the visualisation of DNA fragments

Gels were used primarily to visualise the products of reactions containing DNA such as enzymatic digestion, or to assess approximate DNA concentration. Unless otherwise stated these were used at a concentration of 1% w/v using a stock prepared by dissolving 2 g agarose in 200 ml 0.5X TBE. Ethidium bromide at a final concentration of 0.4 µg/ml was added to gels as they set. This stain enabled DNA within the gel to be visualised under exposure to long wavelength UV light. The gels were run in the same buffer at 40-100 V. DNA samples were prepared in H₂O with DNA loading buffer at a final concentration of 0.1 v/v. An appropriate DNA ladder was used to assess size and concentration of DNA fragments.
2.4.6 Enzymatic digestion (restriction) of DNA molecules

The digestion of DNA was generally undertaken on molecules which had previously been purified using an appropriate QIAgen kit and dissolved in the suppliers EB buffer. The desired quantity of this DNA solution was then further diluted in autoclaved, distilled water and mixed with 0.1 volumes of 10X reaction buffer provided by the enzyme supplier. Where two enzymes were used simultaneously an appropriate buffer was identified for the reaction by referring to the supplier’s instruction as to which buffers were compatible with which enzymes. 1 μl of enzyme stock was used per 10 μl of reaction volume. This was between 2 - 10 U of enzyme depending on that measured by the supplier. Reactions were incubated for 1 to 2 hours at the temperature recommended by the supplier, depending on the amount of DNA used in the reaction.

2.4.7 Ligation

DNA to be ligated was prepared by restriction digestion followed by the appropriate purification technique. The relative quantities of plasmid and insert DNA were assessed by agarose gel electrophoresis. Small samples of each component of the ligation reaction were visualised by this technique and their molar ratio approximately calculated. 10 ng of plasmid DNA / kb were mixed with a 1: 2-6 molar ratio of insert DNA, and the solution was incubated at 65 °C for 5 minutes, before being placed on ice. After a brief time, the appropriate volume of 5X T4 ligase buffer and 1 μl of T4 ligase (1 U / μl) was added per 10 μl of reaction. Sterile distilled water was used to bring the reaction to the final volume...
required in the reaction (10 - 20 µl). Ligation reactions were carried out at 18 °C overnight.

2.4.8 Preparation of competent *Escherichia coli* DH5α cells

Unless otherwise stated, plasmids created in this study were first introduced into competent *E. coli* DH5α following ligation. These cells were obtained from an existing laboratory stock stored frozen at -80 °C. This stock culture was used to prepare aliquots of competent cells ready for use. This was achieved by first streaking the laboratory stock on to a fresh LB plate. After overnight growth at 37 °C a single colony from this plate was selected and inoculated into 5 ml LB broth. This culture was grown overnight at 37 °C and the following morning 2.5 ml was used to inoculate 250 ml of fresh LB broth. This new culture was incubated at 37 °C until the cells had grown to an optical density OD$_{600}$ = 0.3-0.4. The culture was then transferred to five sterile 50 ml Falcon tubes, and incubated on ice for 1 hour, before being centrifuged at 6000 rpm for 10 minutes at 4 °C. Following this initial centrifugation the supernatant was discarded and the pellets were resuspended in 8 ml ice cold 100 mM CaCl$_2$ and combined into a single sterile 50 ml Falcon. The cells were incubated on ice for 20-60 minutes and then centrifuged again at 6000 rpm for 10 minutes at 4 °C. Following this second centrifugation the cells were resuspended in 8 ml 100 mM CaCl$_2$ containing glycerol at a final concentration of 20% v/v. 200 µl aliquots were taken from this solution, transferred
to sterile Eppendorf tubes, and frozen in liquid nitrogen by rapid immersion. These aliquots were then stored frozen at -80°C until use or for a maximum of four months.

2.4.9 Transformation of competent *Escherichia coli* DH5α cells by heat shock

Competent cells were prepared and stored as described above. When required for transformation, frozen aliquots were thawed on ice for 30 minutes prior to use. Following this, the ligation reaction to be cloned was added to the cells and the solution was again incubated on ice for 30 minutes. The tube was then placed in a water-bath set at 42°C, and incubated for 1.5 minutes. After this "heat shock", 1 ml LB was added aseptically by pipette to the tube and it was incubated in a different water-bath set at 37°C for 1 hour. The tube was then removed from the water-bath, briefly vortexed, and dilutions of the cell suspension spread on to an LB plate containing an appropriate antibiotic to select for positive transformants. The remainder of the cell solution in the tube was then centrifuged at 13000 rpm for 1 minute to form a cell pellet. Sufficient of the supernatant was removed to leave approximately 100 μl, into which the cell pellet was then dispersed by pipette. These cells were then spread on to another selective LB plate.
Chapter 2  

Materials and Methods

2.4.10 Isolation of *C. jejuni* genomic DNA

The method for genomic DNA preparation was adapted from Ausuble *et al.*, (1994). *C. jejuni* strains were grown overnight on Mueller Hinton agar under microaerobic conditions at 37 °C for 24 hours. The growth was harvested into 1 ml of TE (10 mmol Tris-HCl; 1 mmol/l EDTA, pH 8) and centrifuged at 15115 rpm. The pellet was resuspended in 567 μl TE and the bacteria were lysed by the addition of 30 μl of 10 % (w/v) sodium dodecyl sulphate (SDS) and 3 μl of proteinase K (20mg/ml) with incubation for one hour at 37 °C. One hundred microlitres of 5 M NaCl was added and the tube contents mixed by inversion. The addition of 80 μl of CTAB/NaCl solution (10 % (w/v) hexadecyltrimethyammonium bromide, 0.7 M NaCl), with thorough mixing and incubation at 65°C for 10 min, resulted in the precipitation of cell wall debris, denatured protein and polysaccharides. This complex was removed by centrifugation. An equal volume of chloroform/isoamylalcohol (24:1) was added to the aqueous layer, mixed and centrifuged. An equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added, mixed and centrifuged. The DNA in the aqueous layer was then precipitated by the addition of 0.6 X volumes of isopropanol. The DNA was pelleted by centrifugation and washed with 70 % (v/v) ethanol. The pellet was air dried for 5-10 min and re-suspended in a 200 μl of water.
2.5 Transformation of *Campylobacter*

### 2.5.1 Preparation of competent cells

*C. jejuni* cells were grown as lawns on two plates of MHA overnight at 37 °C under microaerophilic conditions, and then harvested by using 1 ml M-H broth per plate. Bacteria were pelleted by centrifugation for 5 min at 15000 rpm at 4 °C. Pellets were gently resuspended in 1 ml of ice cold wash buffer (272 mM sucrose, 15% glycerol) and centrifuged. This process was performed three times to wash the cell free of ionic salts. Finally pellets were resuspended in 500 μl of ice cold wash buffer. Cells were aliquoted into 200 μl samples and stored at -80 °C until required.

### 2.5.2 Electroporation

Electroporation cuvettes (Bio-Rad Laboratories Ltd, Hertford, UK) with a 2 mm gap were cooled on ice and a 200 μl aliquot of competent cells thawed on ice. 5 μl of DNA was added to the cells, mixed, and the mixture then placed into an ice cold electroporation cuvette. Electroporation was conducted at 2.5 kV, 200 Ohms, 25 μF (time constant >4 msec). After application of the pulse the cuvette was flushed twice with 100 μl SOC and cells then spread gently onto a non-selective MH agar plate. The plates were incubated at 37 °C for 5 h. Cells were then harvested from the recovery plate, using 400-500 μl M-H broth, and then spread onto large Petri dishes containing the selective antibiotic (chloramphenicol 20 μg·l⁻¹). To allow transformants to grow the plates were incubated at 37 °C under microaerophilic conditions for 3-5 days.
2.5.3 Cloning of the \textit{sdaC} gene

The oligonucleotide primers \textit{sdaCl} 5'-GGATCCATCAGCCTTATCACTCAAGTGTC-3'; \textit{sdaC2} 5'-GATATGCAAGGAAATATCCTAGGGTC-3' were designed to amplify a fragment of approximately 1760 bp containing the \textit{sdaC} gene, with the addition of a \textit{BamHI} restriction sites at one end (underlined). The primers designed were based on the published genome of \textit{C. jejuni} (Parkhill \textit{et al.}, 2000), were analysed using the programme Vector NTi 4.0, and synthesized by Sigma-Genosys Ltd (Cambridgeshire, UK). The primers were supplied lyophilized, and were resuspended in H$_2$O to give a 100\textmu M final concentration, then diluted to provide a working stock concentration of 10\textmu M. The PCR reaction mix for the amplification of the gene fragment contained 1 \mu l of each of the appropriate primers i.e. 0.3 \mu M, 1 \mu l of \textit{C. jejuni} genomic DNA, 0.2 \mu l Taq (Sigma), 0.2 \mu l deoxynucleotide triphosphates (dNTPs) (Roche-200 \mu M$^{-1}$) and 2 \mu l 10X buffer (Sigma). The reaction mix was made up to 20 \mu l with the addition of sterile H$_2$O. The reaction was performed using a Gene Amp® PCR system 9700 (Perkin-Elmer applied Biosystems) which involved an initial denaturation period of 3 minutes at 94 °C, then 35 cycles of 45 seconds at 94 °C to denature, 1 minute at 55 °C to anneal and 2 minute at 72 °C. The final cycle included a step of 10 minutes at 72°C. The samples were stored at 4 °C. The resulting PCR fragment was cloned into pBAD-TOPO and the resulting plasmid designated pNAS1.
2.5.4 Construction of a *sdaC* mutant

pJMK30 was digested with *Bam*HI and the 806 bp kanamycin-resistance cassette purified. pNAS1 was digested with *Bgl*II and the resistance cassette ligated into the vector to generate the suicide plasmid pNSA2. pNAS2 was introduced into *C. jejuni* NCTC11168 by electroporation and one kanamycin transformant designated CjNAS1 confirmed by PCR to be a *sdaC* mutant.

2.5.5 Growth curves of a *sdaC* mutant and *aspA* mutant

The ability of a *sdaC* mutant (CjNAS1) and an *aspA* mutant (CjHAR8) to use serine or aspartate respectively and mucin was assessed by monitoring their growth in defined media in the absence or presence of serine, aspartate, sodium pyruvate or mucin. One ml of a culture grown overnight in 20 ml of MH broth at 37°C for 24 hours was inoculated into 50 ml GMEM (25 mM HEPES and 50 μM FeSO₄) supplemented with and without the following compounds serine (20 mM), aspartate (20 mM), sodium pyruvate (20 mM) and mucin (1%). Cultures were grown at 37 °C in microaerobic conditions with shaking. The growth assessed by measuring OD₆₀₀nm at selected time points.
Chapter 3

The development of a defined medium to establish *Campylobacter* nutrition during colonisation

3.1 Introduction

The mechanisms by which *Campylobacter* colonizes its mammalian hosts are poorly understood and are considered to involve mucosal adherence, host cell invasion and toxin production (Ketley, 1997). In particular the factors that contribute to its metabolic versatility are poorly defined (Velayudhan and Kelly, 2002), as are its nutritional requirements in the gut during colonisation and infection. Research into this aspect of *Campylobacter* physiology has been hampered by the lack of a convenient defined medium for the organism. Complete defined media contain complex mixtures of bacterial nutrients, while minimal defined media contain only those nutrients essential to the growth of a given species. These media have been used for the examination of microbial physiology (Lovitt *et al.*, 1987a) and nutrition (Lovitt *et al.*, 1987b). Defined media have also been used to examine nutritional control of the expression of bacterial virulence mechanisms (Bonas *et al.*, 1991; Schulte and Bonas, 1992).
The first defined media to be described for this pathogen was that reported by (Smibert, 1963) which was used to establish vitamin and amino acid requirements. The complete medium contained 18 amino acids. Later, a defined medium was described by (Tenover et al., 1985), that was a modification of an existing medium used for *Neisseria gonorrhoeae*. Whilst this medium was successfully used for auxotyping studies its production was complex involving the preparation of five individual solutions and the addition of fourteen individual components. More recently, MEM α animal cell tissue culture medium has been used as a pre-prepared medium (Baillon et al., 1999; Velayudhan et al., 2004).

It is often assumed that sugars such as glucose are preferred carbon sources *in vivo* (Smibert, 1984; Velayudhan and Kelly, 2002). Most carbohydrate in the colon however is in the form of mucosal polysaccharides, which are degraded by a few anaerobes that dominate the intestinal biota (Hoskins and Boulding, 1981; Corfield et al., 1992). However, *Campylobacter* is asaccharolytic and uses amino acids as a source of carbon and energy, and its genome has revealed the presence of a number of homologues of amino acid-catabolizing enzymes. *Campylobacter* use amino acids instead as carbon sources for growth in the host. Nevertheless, there are few data on which amino acids might be important *in vivo*. *C. jejuni* is recognized to grow at the expense of amino acids *in vitro* and has been shown to utilize serine, aspartate, glutamate, and proline (Leach et al., 1997). Several amino acids can enter central metabolic pathways such as the Kreb’s cycle through conversion to pyruvate followed by anaplerotic sequences which produce cycle intermediates. The amino acid L-serine is characterized by the fact
that several organisms have the ability to introduce it into the central metabolism through pyruvate (Brown et al., 1990; Hofmeister et al., 1993; Novak and Loubiere, 2000). Stark et al. (1997) reported that amino acids such as alanine, arginine, asparagine, aspartate, glutamine, glutamate, proline and serine were mainly consumed from continuous culture medium by H. pylori cells, which is also a member of the epsilon proteobacteria, and suggested that the products of metabolism of these amino acids can be further metabolized as energy sources by enzymes of the TCA cycle. However, C. jejuni only deaminates serine, aspartate, glutamate, asparagine and glutamine (Westfall et al., 1986; Karmali et al., 1986; Tenover and Patton, 1987; Leach et al., 1997).

The mucus layer covering epithelial tissues is recognized as an important source of carbohydrates for saccharolytic bacteria in the intestine where the supply of fermentable carbohydrate is usually limiting (Salyers and Leedle, 1983; Freter, 1983; Macfarlane et al., 1992). Mucus is a complex gel of glycoproteins and glycolipids; the sugar substituents of mucus include N-acetylglucosamine, N-acetylgalactosamine, galactose, fucose, sialic acids, and lesser amounts of glucuronate and galacturonate (Allen, 1984). Mucin can release monosaccharides and other mucosal glycoproteins and these can support the growth of many intestinal bacteria which do not make polysaccharide-degrading enzymes (Hoskins et al., 1985). The study by Chang et al.,(2004) suggested that there are sugars some of which that can be derived from mucin including gluconate, N-acetylglucosamine, N-acetylneuraminic acid, glucuronate, mannose, fucose and ribose that can support growth of E. coli, in vivo.
The aim of this chapter was to develop a convenient defined media for campylobacters and to use this to investigate the possible sources of nutrition including mucin and its components that these pathogens might use during colonisation.
3.2 Results

3.2.1 The characterization of Minimum Essential Medium (MEM) as a suitable defined media for *C. jejuni*

Minimal Essential Medium (MEM) has previously been used as a defined medium for the growth of *C. jejuni* (Baillon *et al.*, 1999; Velayudhan *et al.*, 2004). Initially it was chosen to assess the ability of *C. jejuni* to use mucin, L-serine, sodium pyruvate, D-glucuronic acid, N-acetylneuraminic acid, ribose, glutamic acid, mannose, fucose and D-glucosamine hydrochloride as sole carbon sources.

![Figure 3.1: Growth of *C. jejuni* in MEM is dependent on mucin. MEM containing FeSO₄ (50 μM) was inoculated at time zero with *C. jejuni* NCTC11168 and cultures grown at 37°C in microaerobic conditions with shaking in presence or absence of mucin at the concentrations indicated. This experiment was performed three times on three independent occasions and similar reproducible results obtained.](image_url)
Growth was only apparent in MEM α following the addition of mucin (Figure 3.1). The extent of growth was dependent on the concentration of mucin and it reached an OD$_{600}$ of 0.284, and 0.521 in the presence of 0.5% and 1% mucin respectively. MEM α agar was next used to assess the ability of different C. jejuni and C. coli strains to use mucin as a carbon source. Table 3.1 shows that in the absence of 1% mucin no significant growth was apparent for any strain. When 1% mucin was added all strains were able to grow indicating that the ability to use mucin or its components is widespread amongst C. jejuni and C. coli strains.

Table 3.1: Growth of C. jejuni and C. coli on MEM α (50 μM FeSO$_4$) solid media with or without mucin

<table>
<thead>
<tr>
<th>Species</th>
<th>1% mucin</th>
<th>No mucin</th>
<th>Mueller-Hinton agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni NCTC 11168</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>C. jejuni NCTC 11951</td>
<td>++</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>C. jejuni NCTC 11847</td>
<td>++</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>C. jejuni NCTC 11322</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>C. jejuni NCTC 11828</td>
<td>+++</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>C. coli UA585</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>C. coli NCTC 11350</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>C. coli NCTC 11366</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>C. coli NCTC 12110</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>C. coli NCTC 11438</td>
<td>+++</td>
<td>–</td>
<td>+++</td>
</tr>
</tbody>
</table>

(+++) Excellent growth; (++) good growth (+) poor growth; (-) no growth.
Since the use of MEM α had demonstrated that mucin could be used as a carbon source by *C. jejuni* the same medium was used to test other carbon sources. However, surprisingly, the entire range of carbon sources provided, except mucin, failed to support growth (Figure 3.2). Consequently, the ability of MEM α to support growth of *C. jejuni* in the presence of carbon sources known to be used by the organism was tested. Thus L-serine and sodium pyruvate were assessed as these have previously been reported to support growth of *C. jejuni* (Velayudhan et al., 2004). However, these compounds did not support growth of *C. jejuni* (Figure 3.3) under the conditions used here.

![Figure 3.2: MEM α supports the growth of *C. jejuni* in the presence of mucin but not other carbon sources. MEM α containing FeSO₄ (50 μM) was inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of mucin or other carbon sources at the concentrations indicated. This experiment was performed three times on three independent occasions and similar reproducible results obtained.](image-url)
Figure 3.3: Growth of *C. jejuni* in MEM α does not occur in the presence of L-serine or sodium pyruvate. MEM α containing 50 μM FeSO4 was inoculated with *C. jejuni* NCTC 11168 and cultures incubated at 37 °C in microaerobic conditions in the presence of mucin (1%), sodium pyruvate (20 mM) and L-serine (20mM). This experiment was performed three times on three independent occasions and similar reproducible results obtained.

These results suggested that MEM α was inhibiting the growth of *C. jejuni*. It was considered possible that the pH of the media could change during carbon source metabolism by *C. jejuni* or due to the carbon dioxide in the microaerobic atmosphere dissolving in the medium to form carbonic acid. In the presence of mucin this effect was alleviated by an unknown mechanism. Thus to determine if the pH of the media was responsible for this inhibition, the pH of MEM α was measured after the growth of *C. jejuni* with different carbon sources. The pH ranged from 5.2, when mucin was available, to 3.1 when glutamic acid was provided. For most carbon sources the pH after incubation was lower than 4.0 (Table 3.2).
Table 3.2: The effect of carbon sources on the pH of MEM α media after incubation with C. jejuni.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH reading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucin</td>
<td>5.2</td>
</tr>
<tr>
<td>L-serine</td>
<td>3.9</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>4.1</td>
</tr>
<tr>
<td>Fucose</td>
<td>3.9</td>
</tr>
<tr>
<td>Mannose</td>
<td>3.8</td>
</tr>
<tr>
<td>Ribose</td>
<td>3.8</td>
</tr>
<tr>
<td>N-acetylneuraminic acid</td>
<td>3.7</td>
</tr>
<tr>
<td>Glucosamine hydrochloric</td>
<td>3.6</td>
</tr>
<tr>
<td>Glucuronic acid sodium</td>
<td>3.8</td>
</tr>
<tr>
<td>Glutamic acid solution</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Since C. jejuni is not capable of growth below pH 4.9 (Blaser et al., 1980b) this explains the fact that mucin and not the other carbon sources allowed the growth of C. jejuni.

To control the pH HEPES was added to MEM α to a final concentration of 25 mM. The addition of 25 mM HEPES allowed the growth of C. jejuni in the presence of sodium pyruvate and L-serine (Figure 3.4) as has been described previously (Velayudhan et al., 2004). This confirms that the failure of C. jejuni to grow in MEM α without HEPES was due to a pH below the limits that allow growth of C. jejuni. C. jejuni also grew in MEM α containing HEPES with fucose, mannose, glucosamine hydrochloric, glucuronic acid sodium, glutamic acid solution, ribose and N-acetylneuraminic acid. However, there was also significant growth to an OD$_{600}$ of 0.107 in the control flask with no added carbon source. Consequently, this media is unsuitable for detailed analysis of carbon source utilization by C. jejuni.
Table 3.3: Ingredients of Minimal Essential Medium (MEM) α and Glasgow minimal medium

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>Molarity (mM)</th>
<th>COMPONENTS</th>
<th>Molarity (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino Acids</strong></td>
<td></td>
<td><strong>Amino Acids</strong></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>0.66700</td>
<td>L-Arginine hydrochloride</td>
<td>0.19900</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>0.28100</td>
<td>L-Cysteine HCl</td>
<td>0.09900</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>0.49800</td>
<td>L-Glutamine</td>
<td>2.00000</td>
</tr>
<tr>
<td>L-Asparagine-H2O</td>
<td>0.16700</td>
<td>L-Histidine hydrochloride-H2O</td>
<td>0.10000</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>0.22600</td>
<td>L-Isoleucine</td>
<td>0.39700</td>
</tr>
<tr>
<td>L-Cysteine hydrochloride-H2O</td>
<td>0.56800</td>
<td>L-Leucine</td>
<td>0.39700</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0.09900</td>
<td>L-Lysine hydrochloride</td>
<td>0.39900</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>0.51000</td>
<td>L-Methionine</td>
<td>0.10100</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>2.00000</td>
<td>L-Phenylalanine</td>
<td>0.20000</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>0.20000</td>
<td>L-Threonine</td>
<td>0.40000</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0.40000</td>
<td>L-Tryptophan</td>
<td>0.03920</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0.39700</td>
<td>L-Tyrosine disodium salt dehydrate</td>
<td>0.19900</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0.39900</td>
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<tr>
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<td>Sodium Phosphate monobasic</td>
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MEM α contains a variety of amino acids (Table 3.3) which could potentially be used as a carbon source by *C. jejuni* and the utilization of these could explain the growth of this organism in the absence of an added carbon source. Indeed, this medium contains both pyruvate and serine, which have been shown previously to support *C. jejuni* growth. Thus Glasgow minimal medium (MacPherson and Stoker, 1962; House, 1964) which contains significantly less amino acids (and no pyruvate or serine) than MEM α (Table 3.3) was assessed as a defined media for *C. jejuni*.
Figure 3.4: Growth of *C. jejuni* in defined MEM α media with HEPES (25mM) supplemented with various carbon sources (all 20mM except mucin) as indicated. Each flask contained FeSO₄ (50 µM) and was inoculated at time zero, with *C. jejuni* NCTC11168. Cultures were grown at 37 °C in microaerobic conditions with shaking. This experiment was performed three times on three independent occasions and similar reproducible results obtained.
3.2.2 Characterization Glasgow Minimum Essential Medium (GMEM)

The ability of GMEM containing HEPES and FeSO₄, supplemented with 20 mM pyruvate, and 20 mM serine to support the growth of *C. jejuni* is illustrated in Figure 3.5. The wild type grew well with pyruvate and serine as the primary carbon source in GMEM. However, in contrast to MEM α media growth was not detected in the absence of added carbon sources suggesting that GMEM is better suited for detected characterization of carbon source utilization by *C. jejuni*.

The improved nature of GMEM for characterizing carbon source utilization is clearly shown in Figure 3.6. *C. jejuni* cannot catabolise glucose probably because it lacks a gene that could encode a 6-phosphofructokinase (Velayudhan and Kelly, 2002). It also appears not capable of catabolising mannose because it also lacks hexokinase and phosphomannose isomerase (Parkhill *et al.*, 2000). Nevertheless, when mannose was provided as a carbon source in MEM α it clearly stimulated growth compared to the control experiment with no carbon source. An interpretation of this result might be the utilization of mannose as a carbon source. However, when the same experiment was performed in GMEM growth did not appear to be stimulated by mannose thus the use of GMEM confirms that *C. jejuni* is not able to catabolise mannose and therefore the apparent growth in MEM α is not due to catabolic activities.
Figure 3.5: Growth of *C. jejuni* in GMEM with HEPES and containing FeSO₄ (50 μM). The medium was inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37°C in microaerobic conditions with shaking in presence of pyruvate and serine at the concentrations indicated.

Figure 3.6: A comparison of the growth of *C. jejuni* in GMEM and in MEM α containing FeSO₄ (50 μM) and mannose (20mM). The media were inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37°C in microaerobic conditions with shaking in the presence or absence of mannose as indicated.
A comparison of the growth of *C. jejuni* in GMEM and MHB was investigated by using viable plate counts. Data presented in Figure 3.7 shows the growth of *C. jejuni* in MHB under the conditions used here. It can be seen that the logarithmic growth phase for *C. jejuni* count began after 3 hrs incubation period and continued for 43 hours. Then the microorganism reached the stationary phase after 54 hrs with the viable count of 1.7 x 10^8 cfu ml⁻¹. Growth of *C. jejuni* in GMEM was similar but when the culture reached the stationary phase after 54 hours the viable count was 6.9 x10^6 cfu ml⁻¹.

### 3.2.3 The growth of different strains of *C. jejuni* and *C. coli* in GMEM

GMEM had been shown to be a suitable media for suitable defined media for studying the growth of *C. jejuni* NCTC 11168. To test its general utility for campylobacters the growth of five strains of *C. coli* and six strains of *C. jejuni* was assessed in this medium and MHB. The growth of all strains in (Figure 3.8) was similar, with NCTC 11168 growing to the highest OD₆₀₀ and NCTC 11322 growing least well. There also appeared to be no difference between the growths of the two *Campylobacter* species. When the strains were introduced into GMEM (Figure 3.9), there were more marked differences in growth. NCTC 11350 failed to grow above an OD₆₀₀ of 0.1, whilst NCTC 11438, NCTC 11951, NCTC 11847 and NCTC 11322 grew better but still only attained an OD₆₀₀ of 0.2 or less. The other strains, NCTC 11437, NCTC11366, NCTC 11828, NCTC 12110, NCTC 11351 and NCTC 11168 grew better and achieved increasing levels of growth in this order. Whether a species was *C. jejuni* or *C. coli* did not appear to influence growth in GMEM.
Figure 3.7: A comparison of the growth of *C. jejuni* in MHB and GMEM supplemented with sodium pyruvate (20mM) and HEPES (25mM). Growth was assessed by plate counts. Cultures were grown at 37 °C in microaerobic conditions in presence of FeSO$_4$ (50 μM). This experiment was performed three times on three independent occasions and similar reproducible results obtained.
Figure 3.8: Growth of strains of *Campylobacter* in MHB media. The medium was inoculated at time zero and cultures grown at 37 °C in microaerobic conditions with shaking. This experiment was performed three times on three independent occasions and similar reproducible results obtained.
Figure 3.9: Growth of different strains of *Campylobacter* in GMEM containing FeSO$_4$ (50 $\mu$M), HEPES 25 $\mu$M and sodium pyruvate (20mM). The media were inoculated at time zero and cultures grown at 37 $^\circ$C in microaerobic conditions with shaking. This experiment was performed three times on three independent occasions and similar reproducible results obtained.
Strains NCTC11350, NCTC11438 and NCTC 11951 grew poorly in GMEM and it was decided to investigate the reason for this. To determine whether this was due to auxotrophy, the strains were grown in GMEM to which different amino acids had been added. When this was carried out with NCTC 11350, the addition of serine or glutamine, but no other amino acid, allowed significant growth to occur (Figure 3.10) suggesting that this strain had a requirement for these two amino acids. When these two amino acids were used to supplement GMEM in which NCTC 11438 and NCTC 11951 were growing, the growth of NCTC 11438 was greatly stimulated and that of NCTC 11951 partially stimulated (Figure 3.11). These results suggest, that as noted by Tenover et al., (1985) that certain strains of campylobacters are auxotrophic, and provided that these auxotrophies are satisfied by the addition of additional amino acids, GMEM is a broadly applicable defined media for campylobacters.
Figure 3.10: Growth curve of *C. coli* NCTC11350 in GMEM to which various amino acids had been added. GMEM containing FeSO₄ (50 μM) with HEPES (25mM) was inoculated at time zero, with *C. coli* 11350 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of sodium pyruvate (20mM). This experiment was performed three times on three independent occasions and similar reproducible results obtained.
Figure 3.11: Growth of diverse strains of Campylobacters, *C. coli* NCTC11350, *C. coli* NCTC11951 and *C. coli* NCTC11438 in GMEM with HEPES 25 mM and FeSO₄ (50 μM) in the presence of various acids. The medium was inoculated at time zero and cultures grown at 37 °C in microaerobic conditions with shaking in presence of L-Serine and Glutamine at the concentrations 20 mM. This experiment was performed three times on three independent occasions and similar reproducible results obtained.
MEM α media has previously been shown to be deficient in iron (Baillon et al., 1999) and iron needs to be added to support *C. jejuni* growth. To determine whether this was the case for GMEM, the growth of *C. jejuni* NCTC 11168 was determined in presence of sodium pyruvate in GMEM medium with or without added FeSO₄. Growth curves thus obtained are shown in (Figure 3.12). In iron-supplemented medium, growth of *C. jejuni* NCTC 11168 was apparent. However, in GMEM media without iron the growth of *C. jejuni* NCTC 11168 was greatly diminished. The final OD₆₀₀ levels measured in this experiment were 0.533 in GMEM with iron and 0.151 without iron. Thus GMEM is iron deficient and this element needs to be added to permit growth of *C. jejuni*.

Figure 3.12: Growth of *C. jejuni* in GMEM with or without FeSO₄ (50 µM). The media was inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of sodium pyruvate (20mM). This experiment was performed three times on three independent occasions and similar reproducible results obtained.
3.2.4 Characterisation of amino acids as carbon sources for C. jejuni NCTC11168

C. jejuni is known to grow at the expense of amino acids and has been reported to utilize serine, aspartate, glutamate and proline (Leach et al., 1997). It also possesses several enzymes for amino acid deamination (Parkhill et al., 2000) and thus amino acids are likely to be major carbon sources.

The growth of C. jejuni was studied in GMEM with various amino acids provided as potential carbon sources. Among the carbon sources tested in Figure 3.13, L-serine was found to support C. jejuni growth and has been shown previously to be preferentially utilized compared to other amino acids (Leach et al., 1997). The highest degree of growth was measured after 102.25 hours incubation (0.467 OD₆₀₀), whereas L-proline, glutamic acid, L-valine, L-glutamine, L-histidine, L-tyrosine, L-asparagine cysteine, and glycine all give rise to growth but this was modest compared to L-serine after 30 hours and OD₆₀₀ was 0.149, 0.082, 0.135, 0.132, 0.096, 0.057, 0.060 and 0.110 respectively. On the other hand, C. jejuni was unable to grow when L-arginine, L-methionine, L-lysine, L-threonine, L-alanine and L-phenylalanine were provided as carbon sources.

3.2.5 Characterisation of sugars and derivatives as a source of carbon for C. jejuni

This study has shown that C. jejuni can utilise mucin as a carbon source. Similarly, E. coli has been shown to utilise nutrients acquired from mucus (Sweeney et al., 1996; Chang et al., 2004). Thus, GMEM was used to assess whether the sugar constituents of
mucin could be utilised by *C. jejuni* (Figure 3.14). Of the seven of the sugars tested only fucose, ribose (although the OD$_{600}$ declined after 50 hours incubation) and D-glucosamine hydrochloride supported growth but growth in the presence of these sugars was much reduced compared to that seen in the presence of pyruvate.

Figure 3.13: Growth of *C. jejuni* in GMEM with various amino acids. GMEM containing FeSO$_4$ (50 μM) with HEPES (25mM) was inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking. This experiment was performed three times on three independent occasions and similar reproducible results obtained.
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Figure 3.14: Growth of C. jejuni in GMEM with different sugars and derivatives. GMEM containing FeSO₄ (50 μM) was inoculated at time zero with C. jejuni NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of mucin and compounds at the concentrations indicated (20mM). This experiment was performed three times on three independent occasions and similar reproducible results obtained.
3.3 Discussion

The present study investigated the use of an improved defined medium for studying the utilization of carbon sources by *C. jejuni*. MEM α animal cell tissue culture media has most recently been used as convenient pre-prepared defined media (Baillon *et al.*, 1999; Velayudhan *et al.*, 2004). Initially this study sought to use MEM α to establish the carbon sources utilized by *C. jejuni*. However, whilst MEM α supported growth in the presence of mucin as a sole carbon source, no growth was detected when serine or pyruvate were provided singly. These are known to be utilized as carbon sources (Velayudhan *et al.*, 2004). The inability of MEM α to support growth in the presence of these compounds was attributed to the acidification of the medium to a pH below the limits of growth for *C. jejuni*. This effect was alleviated by addition of 25 mM HEPES and hereafter all media used contained this buffer to prevent medium acidification. However, whilst this media was shown to be suitable for demonstrating the use of mucin and other carbon sources, in the absence of provided carbon sources limited growth still occurred and this precluded its use for further studies. Consequently, an alternative defined media was sought.

Glasgow minimum essential medium (GMEM) (MacPherson and Stoker, 1962; House, 1964) was chosen as an alternative to MEM α because it contains significantly less amino acids (and no pyruvate or serine). GMEM was also found to be iron-limiting but the addition of 50μM FeSO₄ allowed growth. GMEM offers a significant improvement over MEM α because, in contrast, no growth at all was apparent when no additional carbon
sources were provided. The availability of carbon sources (serine and pyruvate) in MEM α, and the level of growth that this permits, can lead to the misidentification of potential carbon sources. For example, (Velayudhan and Kelly, 2002) have demonstrated that C. jejuni cannot catabolise glucose probably because it lacks the gene that could encode a 6-phosphofructokinase kinase. Because it also lacks hexokinase and phosphomannose isomerase it also should not be capable of catabolising mannose (Parkhill et al., 2000). Nevertheless, in this study when mannose was provided as a carbon source in MEM α it clearly stimulated growth compared to the control experiment with no carbon source. An interpretation of this result might be the utilization of mannose as a carbon source. However, when the same experiment was performed in GMEM, growth did not appear to be stimulated by mannose thus the use of GMEM confirms that C. jejuni is not able to catabolise mannose and therefore the apparent growth in MEM α is not due to catabolic activities. C. jejuni produces an extracellular polysaccharide containing mannose (Muldoon et al., 2002) and stimulated growth in MEM α may have been due to the incorporation of the added mannose into this polysaccharide, and subsequently, the diversion of carbon from the other carbon sources in MEM α away from polysaccharide production into other essential pathways.

Whilst most experiments with GEMM were carried out with C. jejuni NCTC 11168, this defined media was also tested for generally utility, by studying the growth of six and five C. jejuni and C. coli strains respectively. Six strains of Campylobacter grew well in the media suggesting that its use need not only be confined to the study of NCTC 11168. In the case of two strains that failed to grow, their growth was stimulated by the addition of
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the amino acids, either serine or glutamine. This suggests, as noted by Tenover et al., (1985), that certain strains of campylobacters are auxotrophic, and provided that these auxotrophies are satisfied by the addition of additional amino acids, GMEM is a broadly applicable defined media for most campylobacters.

GMEM was used to investigate the use of various amino acids by C. jejuni. L-serine and pyruvate were observed to support growth. This supports the findings of earlier studies (Leach et al., 1997; Velayudhan et al., 2004). While L-proline, glutamic acid, L-valine, L-glutamine, L-histidine, L-tyrosine, L-asparagine, L-cysteine, and L-glycine stimulated growth, this was not to the same extent as that seen for L-serine. On the other hand, C. jejuni was unable to grow when L-arginine, L-methionine, L-lysine, L-threonine, L-alanine and L-phenylalanine were provided as carbon sources. Aspartate was not included in this chapter because its use as a carbon source is shown in the next chapter. Notably, this study has shown that aspartate, glutamate, serine and proline support the growth of C. jejuni 11168. Coupled with the finding that aspartate, glutamate, serine, and proline are in fact the most abundant amino acids present in chicken excreta (Parsons et al., 1983) this suggests that these amino acids are important carbon sources in vivo.

In general the mucin layer has a protective role in overlying the epithelial cells (Schroten et al., 1992; de Repentigny et al., 2000). In addition, the mucous layer also contains other important elements such as phospholipids which may have an effect on bacterial translocation (Usui et al., 1999). Mucus is a complex gel of glycoproteins and glycolipids (Allen, 1984). The primary function of intestinal mucin is to provide a
selective diffusion barrier against penetration of the mucosa by bacteria, toxins, and dietary components, but mucin degradation and utilization by the intestinal bacterial flora is well established (Lee et al., 1986b; Hugdahl et al., 1988; Corfield et al., 1992). Previously it has been shown that mucin preparations enhance the growth of some bacteria (Bradshaw et al., 1994). For example (Taneera et al., 2002) examined five species of Helicobacter pylori and found that porcine gastric mucin stimulated the growth of all. This study demonstrated that C. jejuni can utilize mucin when it is provided as a sole carbon source since growth in the two defined media was enhanced by its addition.

The sugar substituents of mucus include N-acetylgalactosamine, N-acetylglucosamine, fucose, galactose, sialic acids, and smaller amounts of glucuronate and galacturonate. The majority of enteric bacteria require a carbohydrate for growth, and utilisation of these is assumed to be the mode of metabolism used by most species (Salyers and Leedle, 1983). Most carbohydrate in the colon is in the form of mucosal polysaccharides, which are degraded by a few anaerobes that dominate the intestinal biota. The monosaccharides released from mucin and other mucosal glycoproteins support the growth of many intestinal bacteria such as E. coli, which does not make polysaccharide-degrading enzymes (Corfield et al., 1992). Based upon whole genome expression profiling Chang et al., (2004) have suggested that there are seven sugars including gluconate, N-acetylglucosamine, N-acetylneuraminic acid, glucuronate, mannose, fucose and ribose that can promote the growth of E. coli, in vivo. Amongst the sugars tested in this study only fucose, ribose and D-glucosamine hydrochloride
supported growth of *C. jejuni* but this was much reduced in comparison to that seen in the presence of pyruvate. Pyruvate has been shown to be the best carbon source to enhance *C. jejuni* growth (Velayudhan and Kelly, 2002). Nevertheless, it does demonstrate that *C. jejuni* is able to utilise some of the products of mucin degradation and this is likely to be important during colonisation.
Chapter 4

An investigation into the mechanisms of mucin utilisation in Campylobacter

4.1 Introduction

Mucins are an important element of the gastrointestinal mucosal defense against pathogens (McAuley et al., 2007). They are glycoproteins containing large quantities of galactose and hexosamines with a smaller amount of fucose (Quigley and Kelly, 1995). The high molecular weight mucins are responsible for the viscoelastic properties of the mucous barrier. Also mucins are likely to be major sources of carbohydrate for saccharolytic bacteria growing in the large intestine (Macfarlane et al., 1992). Many intestinal microorganisms use these molecules as carbon, nitrogen, and energy sources (Cohen et al., 1983; McCormick et al., 1988; Wilson and F., 1988; Macfarlane and Gibson, 1991; Macfarlane et al., 2001). Most mucosal pathogens have evolved adhesins for carbohydrates, and many adhesins bind mucin oligosaccharides (Linden et al., 2002). However, mucin needs to be degraded by diverse hydrolytic enzymes into smaller oligomers, monosaccharides, and amino acids before it can be assimilated by intestinal microorganisms. Different culture studies have established in many gut bacteria, synthesis of these enzymes, particularly N-acetyl β-glucosaminidase, β-galactosidase, and
neuraminidase (Macfarlane et al., 1989; Macfarlane and Gibson, 1991; Macfarlane et al., 1997; Linden et al., 2002).

Mucinases and glycosidases are enzymes competent at degrading mucins. Partial or complete degradation of mucin molecules by microbial enzymes is often a primary step in disturbance of defensive mucosal barriers, and a prelude to disease, as these constitute direct interfaces between the inside and outside environments. The probable contribution of mucin degrading enzymes to the pathogenesis of infection is, therefore, not to be underestimated. Mucinases, in particular, may play a very important part in the aetiology of certain infections (McGregor et al., 1994; Howe et al., 1999; Bagriacik and Miller, 1999). Since, in the previous chapter, it had been shown that C. jejuni could grow in the presence of mucin, when it was provided as a sole carbon source, and also using its degradation products, one of the aims of this chapter was to establish whether the bacterium possessed any enzymes capable of mucin degradation.

L-serine is nonessential amino acid that can be introduced into central metabolism via pyruvate (Brown et al., 1990; Hofmeister et al., 1993; Novak and Loubiere, 2000). Velayudhan et al., (2004) observed that C. jejuni can utilise L-serine and that the two products of serine deamination, pyruvate and ammonia, provide forms of carbon and nitrogen that can feed directly into the central metabolism. In addition, since mutants deficient in serine catabolism, colonize a chicken model poorly, this amino acid might provide an important carbon source in vivo (Velayudhan et al., 2004).
SdaA catalyzes the deamination of serine to pyruvate and ammonia, both of which can be readily assimilated. The *sdaA* gene in *C. jejuni* 11168 is the downstream gene of a two-gene operon of which the first gene (*Cj1625c*) encodes a high affinity putative serine transporter, homologous to *E. coli* SdaC (Grabowski *et al.*, 1993; Shao *et al.*, 1994; Velayudhan *et al.*, 2004). However, in *C. jejuni* serine transport it also carried out by a low-affinity L-serine-specific transporter since there are residual transport kinetics in a *sdaC* mutant (Velayudhan *et al.*, 2004).

Aspartate another amino acid is deaminated to fumarate by AspA, which can then be metabolized through the citric acid cycle or used as an electron acceptor. In *C. jejuni*, the putative aspartate-ammonia lyase gene, *aspA* (*Cj0087*), is downstream of the gene encoding an anaerobic C₄-dicarboxylate transporter (*Cj0088*) (Woodall *et al.*, 2005). Therefore, *in vivo*, *C. jejuni* aspartate utilization may also be linked to C₄-dicarboxylate utilization (Woodall *et al.*, 2005). Recently, Leon-Kempis Mdel (2006) identified that the transport function of PEB1a, and the PEB1 system, is of crucial importance to the ability of *C. jejuni* to catabolise aspartate in the gut.

Since serine and aspartate might be products of mucin breakdown and thus a means by which *C. jejuni* could acquire nutrition from this substance, the second aim of this chapter was to determine whether *C. jejuni* mutants, defective in serine or aspartate catabolism could grow in the presence of mucin.
4.2 Results

4.2.1 Generation and characterization of a sdaC mutant strain

SdaC is a recently described highly specific serine transporter while SdaA is a serine ammonia-lyase which has the ability to introduce serine into central metabolism via pyruvate. These two components are necessary for C. jejuni to use serine as a carbon source (Velayudhan et al., 2004). Therefore it was decided to construct a sdaC mutant to determine if GMEM could be used to analyse the phenotype of the sdaC mutant and also to find out whether the inability to use serine affected the ability of C. jejuni to utilize mucin, as serine is a major component amino acid of mucin (Karnes et al., 1991).

4.2.1.1 PCR amplification

The sdaC gene of C. jejuni NCTC11168 and flanking regions were amplified using the oligonucleotide primers SdaC1 and SdaC2. The amplification of the sdaC gene was successful yielding a fragment of 1760 bp, which was the estimated band size expected (Figure 4.3), as shown in Figure 4.1.
Figure 4.1: An 0.8% agarose gel showing the amplification of the \textit{C. jejuni} NCTC11168, \textit{sdaC} gene by PCR using primers SdaC1 and SdaC2. (Lane 1) 1Kb ladder, (Lane 2) DNA target alone, (Lane 3) PCR reaction using SdaC1 and SdaC2 (1/10 of target dilution), (Lane 4) PCR reaction using SdaC1 and SdaC2 (1/100 of target dilution), (Lane 5) No target DNA.
4.2.1.2 Cloning the \textit{sdaC} gene fragment using the pBAD TOPO cloning vector

The gene fragment comprising \textit{sdaC} was cloned into the pBAD TOPO vector. pBAD is a linearised plasmid with single terminal 3' deoxythymidine (T) residues which allows efficient ligation with PCR inserts, as the PCR products have terminal single deoxythymidines (A) added to the 3' end by \textit{Taq} polymerase. The ligation was mixed transformed into chemically competent \textit{E. coli} cells (DH5α). Cells containing the vector were selected using LB agar containing ampicillin, only cells containing the vector were able to grow on the media as pBAD contains a gene encoding for ampicillin antibiotic resistance. A mini plasmid preparation was carried out to screen the plasmids for possession of the desired plasmid.

Three colonies were selected and the plasmids contained within were purified using the Miniprep kit. As can be seen from Figure 4.2 the three transformants contained supercoiled plasmid that was larger than the parental vector pBAD TOPO. This is consistent with this plasmid containing the \textit{sdaC} gene fragment.
Figure 4.2: Agarose gel (0.8%) showing a miniprep of selected colonies from LB agar with ampicillin. (Lane 1) 1Kb ladder, (Lane 2) pBAD TOPO, (Lane 3, 4 and 5) plasmids from three separate transformants.
Figure 4.3: Representation of the \textit{sdaC} gene showing the binding sites for primers SdaC1 and SdaC2 and \textit{BglII} sites.
There are two *BamH1* sites in the pBAD plasmid (these are close together and do not liberate a fragment visible on agarose gels), and another incorporated into primer *sdaC1*. Thus if the *sdaC* fragment is cloned in one orientation digestion with *BamH1* will liberate a 1760 bp fragment. In the opposite direction the fragments liberated will be too small to be observed. As can be seen in Figure 4.4 the plasmids in lanes 3 and 5 produced a band of 5996 bp and thus contain the insert in one direction. Plasmid in lane 4 contained the insert in the opposite direction since digestion with *BamH1* liberated a 4000 bp fragment which is the pBAD TOPO backbone and a 1760 bp fragment which is the *sdaC* insert. This plasmid was designated pNAS1.

![Figure 4.4: BamH1 restriction digests of pBAD derivatives resolved on an 0.8% agarose gel. (Lane 1) 1Kb ladder, (Lane 2) pBAD TOPO digested with BamH1, (Lane 3-5) plasmids isolated from transformants digested with BamH1.](image-url)
4.2.1.3 Insertion of kanamycin resistance cassette into \textit{sdaC}

Digestion of pJMK30 with \textit{BamHI} liberates a 1490 bp kanamycin-resistance cassette (Figure 4.5). Digestion of pNAS1 with \textit{Bg/III} liberates a 806 bp fragment internal to \textit{sdaC} (Figure 4.5). pNAS1 was digested with \textit{Bg/III} and the 5880 bp fragment containing the plasmid backbone and terminal regions of the \textit{sdaC} gene gel purified. This fragment was ligated to the purified 1490bp \textit{BamHI} fragment from pJMK30.

\begin{center}
\includegraphics[width=0.5\textwidth]{figure4.5.png}
\end{center}

\textbf{Figure 4.5:} Agarose gel 0.8\% showing, (Lane 1) 1Kb ladder, (Lane 2) pJMK30 digested with \textit{Bam HI}, (Lane 3) pNAS1 digested with \textit{Bg/III}. 
The ligation was introduced into *E. coli* DH5α by chemical transformation and ampicillin and kanamycin resistant transformants selected. Plasmids from three such transformants were purified by miniprep and digested with *Bam*HI to confirm the insertion of the kanamycin resistance cassette (Figure 4.6). Whilst digestion of pNAS1 liberated a 1760 bp fragment digestion of these plasmids liberated a 2444 bp fragment. This is consistent with the introduction of the kanamycin cassette into pNAS1.

Figure 4.6: Agarose gel 0.8% showing plasmids digested with *Bam*HI. (Lane 1) 1Kb ladder, (Lane 2) pNAS1, (Lane 3-5) Plasmids from ampicillin and kanamycin resistant transformants.
PCR was used to determine the orientation of the kanamycin cassette in the derivatives of pNAS1. The orientation of the SdaC1 and SdaC2 primers is shown in Figure 4.3. Kan1 and Kan2 are primers specific for the gene encoding kanamycin resistance and are designed such that Kan1 will extend from the 5' end of the gene, and Kan2 is to extend in the opposite direction and from the 3' end of the gene. If the kanamycin resistance cassette is orientated with the same transcriptional polarity as the sdaC gene then primer combinations SdaC2 with Kan2, and SdaC1 with Kan1 would be expected to generate PCR products. However, these combinations did not generate products in PCR reactions (data not shown) and instead primer combinations SdaC2 with Kan1 and SdaC1 and Kan2 generated products (Figure 4.7). This is consistent with the kanamycin resistance
cassette being orientated such that it is transcribed in the opposite direction to \textit{sdaC} in all three of the plasmids examined. One of these plasmids was designated pNAS2.

### 4.2.1.4 Construction of a \textit{C. jejuni sdaC} mutant using the suicide plasmid pNAS2

To construct a \textit{C. jejuni sdaC} mutant the suicide plasmid pNAS2 was introduced into \textit{C. jejuni} NCTC11168 by electroporation and transformants selected on the basis of kanamycin resistance. The genotype of five kanamycin resistance colonies was assessed using PCR primers \textit{sdaCl} and \textit{sdaC2} (Figure 4.8). All five transformants yielded fragments consistent with the insertion of the 1480bp kanamycin resistance cassette and were thus \textit{sdaC} mutants. The mutant represented in lane 6 (Figure 4.8) was designated CJNAS1.

![Figure 4.8: The outcome of PCR using primers sdaCl and sdaC2 when DNA from putative sdaC mutants was used as target. (Lane 1) 1Kb ladder, (Lane 2-6), target DNA from putative sdaC mutants (Lane7), target DNA from the parental strain NCTC 11168.](image)

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4.2.1.5 Effect of the *sdaC* mutation on the growth of *C. jejuni* in Glasgow Minimum Essential Medium containing L-serine

The phenotype of the *sdaC* mutant CJNAS1 was assessed by monitoring its growth in defined media in the presence or absence of serine. As can be seen in Figure 4.9, growth of CJNAS1 and the wild type was comparable in the presence of mucin and pyruvate. However, whilst the wild type grew well in the presence of serine (final OD$_{600}$ of 0.467) the *sdaC* mutant failed to grow (final OD$_{600}$ was 0.035). Thus *sdaC* is essential for serine utilization by *C. jejuni* but not mucin.

![Figure 4.9](image_url)

Figure 4.9: Growth of *C. jejuni* NCTC11168 wild type and mutant type (CjNAS1) in GMEM containing FeSO$_4$ (50 μM). Cells were inoculated at time zero and cultures grown at 37 °C in microaerobic conditions with shaking using the carbon sources shown. This experiment was performed three times on three independent occasions and similar reproducible result obtained.
4.2.2 Acquisition and characterization of an *aspA* mutant strain

*C. jejuni* has been shown to grow at the expense of amino acids *in vitro* and has been also shown to utilize aspartate (Leach *et al.*, 1997). The gene Cj0087 is predicted to encode an aspartate ammonia lyase (AspA) which would liberate oxaloacetate from aspartate. Since oxaloacetate can be fed directly into central metabolism AspA might be essential for the ability of *C. jejuni* to use aspartate as a carbon source. To test the utility of GMEM media again, an *aspA* mutant CJHAR8 generated by H. Talbot (University of Surrey) was grown in the presence or absence of aspartate.

![Graph showing growth comparison](image)

Figure 4.10: Comparison of the growth of *C. jejuni* NCTC 11168 wild type and mutant type (CjHAR8) in GMEM containing FeSO₄ (50 μM). The media were inoculated at time zero and cultures grown at 37 °C in microaerobic conditions with shaking in the presence of carbon sources as indicated. This experiment was performed three times on three independent occasions and similar reproducible result obtained.
As is shown in Figure 4.10 the wild type and mutant grew well in the presence of mucin and pyruvate. However, whilst the wild type grew in GMEM media containing aspartate, the \textit{aspA} mutant did not. Thus AspA is clearly essential for utilization of aspartate by \textit{C. jejuni} but not mucin.

\subsection*{4.2.3 Plate based protease and mucinase assays}

To determine whether \textit{C. jejuni} produced extracellular proteases or mucinases able to degrade mucin and proteins two crude plate based assays were attempted. Plates of GMEM agar containing either 2\% skimmed milk or 2\% mucin were prepared. In both types of plates the media was visibly clouded by the added proteinaceous material and it was hoped that if broad activity proteases were produced that clearing zones would appear. However, when wild-type \textit{C. jejuni} and \textit{C. coli} were streaked onto plates no clearing zone appeared in the plates even after five days of incubation at 37°C in microaerobic jars. Thus either campylobacters do not produce these enzymes or this type of assay cannot detect them.

\subsection*{4.2.4 Effect of enzyme production in \textit{C. jejuni} growth}

To investigate whether \textit{Campylobacter} produced enzymes with the potential to degrade mucin during growth in GMEM media with 1\% mucin, culture supernatants of \textit{C. jejuni} were assessed for glycosidase production using the \textit{p}-nitrophenol- linked substrates, \textit{p}-
nitrophenol-α-L-fucopyranoside, p-nitrophenol-α-D-glucopyranoside, p-nitrophenol-β-D-glucopyranoside, p-nitrophenol-α-D-galactopyranoside and p-nitrophenol-β-D-galactopyranoside all of which represent carbohydrate residues commonly found on mucin (Tzortzis et al., 2003). The results are displayed in Table 4.1 ABC with negative controls in which the supernatant was not added to the reaction. No significant increase in OD$_{400}$ nm was detected when culture supernatants of C. jejuni were incubated in the presence of the p-nitrophenol linked substrates, suggesting that C. jejuni does not possess the glycosides activities that recognise the linked substrates.
Table 4.1: Measuring enzyme activity using p-nitrophenol substrates in *C. jejuni*. Tables A, B and C represent three separate attempts.

<table>
<thead>
<tr>
<th>A Substrate</th>
<th>Incubation time (min)</th>
<th>-ve control</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OD 400nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>p</em>-nitrophenol -α-L-fucopyranoside</td>
<td>0.090</td>
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<tr>
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<td>0.042</td>
<td>0.081</td>
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<tr>
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<tr>
<td><em>p</em>-nitrophenol -β-D-galactopyranoside</td>
<td>0.150</td>
<td>0.088</td>
<td>0.073</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>B Substrate</th>
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<th>10</th>
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<tr>
<td></td>
<td></td>
<td>OD 400nm</td>
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<td></td>
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<tr>
<td><em>p</em>-nitrophenol -α-L-fucopyranoside</td>
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<tr>
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<td>0.053</td>
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<tr>
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<td>0.086</td>
<td>0.053</td>
<td>0.050</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C Substrate</th>
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</tr>
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<tr>
<td></td>
<td></td>
<td>OD 400nm</td>
<td></td>
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</tr>
<tr>
<td><em>p</em>-nitrophenol -α-L-fucopyranoside</td>
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<td>0.016</td>
<td>0.040</td>
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<td>0.034</td>
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<tr>
<td><em>p</em>-nitrophenol -β-D-galactopyranoside</td>
<td>0.086</td>
<td>0.026</td>
<td>0.033</td>
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</tr>
</tbody>
</table>
4.3 Discussion

Recently, serine transport in *C. jejuni* has been shown to be carried out by a transporter encoded by *(SdaC)* and the initial stages of its catabolism by a L-serine dehydratase enzyme *SdaA* (Velayudhan *et al.*, 2004). In this study, a *sdaC* mutant was constructed to determine if GMEM could be used to analyse the phenotype of the *sdaC* mutant and also to find out whether the inability to use serine affected the ability of *C. jejuni* to utilize mucin, as serine is a major component of mucin (Karnes *et al.*, 1991). The *sdaC* mutant CJNSA1 was unable to use serine as a carbon source because of the lack of activity of *SdaC*. However, the mutant was not affected in its ability to grow in the presence of mucin suggesting that mucin does not act as a significant source of available serine. *C. jejuni* has also been shown to utilize aspartate *in vitro* (Leach *et al.*, 1997). The gene *Cj0087* is predicted to encode an aspartate ammonia lyase (*AspA*) which would liberate oxaloacetate from aspartate. Since oxaloacetate can be fed directly into central metabolism, *AspA* might be essential for the ability of *C. jejuni* to use aspartate as a carbon source. To test the utility of GMEM media again, an *aspA* mutant CJHAR8 generated by H. Talbot (University of Surrey) was grown in the presence or absence of aspartate. This study demonstrated that CJHAR8 was not able to use aspartate as carbon source. However, the mutant grew well in the presence of mucin and pyruvate. Thus *AspA* is clearly essential for utilization of aspartate by *C. jejuni* but not for growth in the presence of mucin. Consequently, *C. jejuni* is not using mucin as a source of serine or aspartate and consequently must be acquiring other carbon compounds from this molecule.
Chapter 4  The mechanisms of mucin utilisation in Campylobacter

The glycoprotein nature of small intestinal and colonic mucins makes them susceptible to attack by both glycosidases and proteases. Several species of bacteria indigenous to the human and animal intestinal tracts including Campylobacter have been shown to utilize mucin as a nutrient source (Stanley et al., 1986; Beery et al., 1988; McAuley et al., 2007; Chapter 3 of this thesis). Mucin degradation by bacteria begins with the proteolytic attack on the non-glycosylated regions of the peptide core, yielding mucin glycopeptides. The bacteria then utilize their glycosidic enzymes for cleavage of carbohydrates from the mucin molecule (Deplancke and Gaskins, 2001). These enzymes may act in conjunction with each other to promote complete degradation of the glycoprotein (Stewart-Tull et al., 1986; Dwarakanath et al., 1995). In this context, a protease activity of Vibrio cholerae degraded mucin, causing a reduction in the viscosity (Crowther et al., 1987), and an Entamoeba histolytica cysteine protease activity degraded mucin and rendered it 40% less effective in the inhibition of bacterial penetration of cultured cells (Moncada et al., 2000). A number of bacteria have also been shown to produce glycosidic enzymes and these are commonly assayed for using p-nitrophenol substrates. β-D-galactosidase, N-acetyl-β-D-galactosaminidase, α-fucosidases, and N-acetyl-β-D-glucosaminidase activities have been shown to cleave sugars from mucin oligosaccharides (Howe et al., 1999). α-galactosidase has been known to preferentially transfer galactosyl residues to the primary alcoholic groups of acceptor sugars (Dey and Pridham, 1972). van Laere et al.,(1999) have been observed that a α-gal-acto-oligosaccharides could be formed from melibiose using Bifidobacterium adolescentis α-galactosidase. Also, Mitsuomi and Ohtakara (1988) have shown that transferase activity of α-galactosidase from Streptococcus bovis, which was found to have transglycosylation
activity in the hydrolysis of \( p \)-nitrophenyl \( \alpha \)-D-galactopyranoside (Eneyskaya et al., 1998). The hydrolysis of a different of natural and synthetic substrates methyl \( \alpha \)-D-galactopyranoside, and \( p \)-nitrophenyl \( \alpha \)-D-galactopyranoside, was used to detect \( \alpha \)-D-galactosidase activity *Trichoderma reesei*, (Savel'ev et al., 1996) but no evidence of other activities were detected using \( p \)-nitrophenol-\( \alpha \)-D-galactopyranoside, \( p \)-nitrophenol-\( \beta \)-D-galactopyranoside and \( p \)-nitrophenol-\( \alpha \)-D-glucopyranoside.

Since it had been shown in Chapter 3 that *C. jejuni* could utilize mucin for growth, two methods were used to establish whether this pathogen was able to degrade mucin. In both cases, using a plate based assay for proteineases and mucinases, and using \( p \)-nitrophenol derivatives of sugars no proteolytic or glycosidic activities were detected. Either *C. jejuni* does not possess the enzymes or the assays were not able to detect activities that were present because of difference in the specificity of the activities when matched to the substrates.
An investigation into the mechanisms of peptide transport and utilisation in \textit{Campylobacter}

5.1 Introduction

Previous work in this study had demonstrated that \textit{C. jejuni} was able to utilize mucin as a sole carbon source. Because of the complexity of this molecule it is most likely that this bacterium transports and utilizes the products of mucin degradation some of which will be short peptides.

Peptides serve as an important source of nutrients for most species of bacteria (Abouhamad \textit{et al.}, 1991). There are four main types of transport system involved in the transfer of small molecules across the cell membrane of bacteria, these involve; passive diffusion; facilitated diffusion; group translocation and active transport. It is generally accepted that small neutral peptides may be transported passively across the membrane, but that in general, passive diffusion is considered to be insignificant as a mechanism for peptide uptake (Payne, 1976). It is clear that the predominant mode of uptake of peptides in bacteria is by active transport (Leach and Snell, 1960; Meisler and Simmonds, 1963; De Felice \textit{et al.}, 1973). Whilst most bacterial species have individual transport systems for the majority of amino acids that are closely related in structure
transport of peptides is more complicated as variations can be too great to warrant having a specific transport system for each single peptide. Despite this, certain structural requirements are made by peptide transport systems to allow satisfactory transport into the cell. For example, research shows that a positively charged primary amino group is the minimum structural requirement for uptake by *E. coli* peptide-transport systems (Payne, 1971). Some evidence suggests that the cell membrane acts as a sieve, preventing large peptides from reaching the cytoplasmic membrane, but that these sieves differ for different organisms and strains (Naider et al., 1974). Although few bacterial species other than *Salmonella* and *E. coli* have been studied in detail, examination of transport systems in other organisms has increasingly highlighted similarities between the systems present in various species. This is particularly true of *P. aeruginosa* which although having a complex and unusual cell-envelope structure has been shown to have peptide permeases which resemble model systems found in *E. coli* (Payne and Smith, 1994). In Gram negative bacteria, peptides pass through the outer membrane through pores known as porins. Once they reach this stage, there are three distinct peptide transport systems that are responsible for uptake which have overlapping substrate specificities in enteric bacteria (Higgins and Gibson, 1986). The first group of peptide transports is known as oligopeptide permeases (OPP), the second are tripeptide permeases (TPP) and the third are known as the dipeptides permeases (DPP). These permease systems are the most completely characterised of all the peptide transport systems (Payne and Smith, 1994). The oligopeptide transport system comprises a periplasmic binding protein-dependent system belonging to a class of bacterial periplasmic transport systems called ATPases (Ames and Joshi, 1990). The presence of
the tripeptide permease (Tpp) system was discovered when well characterised opp mutants were shown to still be able to grow on a restricted range of tripeptides (Naider and Becker, 1975). These permeases have a similar specificity to the Opp system and need a positively charged amino terminus. Tripeptide permeases have the highest affinity for hydrophobic tripeptides, in particular those with N-terminal residues, although they can transport dipeptides to some extent (Gibson et al., 1984). The Tpp system has a relatively minor role in normal growth situations but is expressed strongly in anaerobic conditions for example in the anaerobic environment of the mammalian gut (Payne and Smith, 1994). The dipeptide permease (Dpp) system, like the Tpp system has a great affinity for dipeptides, but unlike the Tpp system, they cannot transport peptides larger than this (Payne and Smith, 1994). The Dpp system has stricter structural requirements than the Opp or Tpp system and is inhibited if the C-terminal α-carboxyle is modified or lost. Even so, dipeptides with modified peptide bonds and extended backbones are transported well (Morley et al., 1983). Although, dipeptide permeases can transport varied dipeptides, their uptake is influenced greater by side chain modifications than is the case with Opp (Alves and Payne, 1980). \textit{Lactococcus lactis} possesses two transport systems suitable for both di- and tri-peptides, both differing from systems found in \textit{E. coli}. These systems are proton motive force-dependent carrier proteins, and are secondary transport systems to the binding protein dependent ATP driven peptide transport systems, which are seen in the majority of organisms (Hagting et al., 1994). Whereas the Opp of \textit{L. lactis} is considered essential for nutrition, the di-tripeptide transport systems are thought to regulate expression of genes involved in nitrogen metabolism (Marugg et al., 1995; Guedon et al., 2001b; Guedon et al., 2001a).
The Dpp system of *L. lactis*, like that of *E. coli*, requires a free N-terminal α-amino group within the ligand for uptake, as shown by *E. coli* Opp (Sanz *et al.*, 2003).

Peptides may be degraded intracellularly or extracellularly and peptidases which can be categorised into two groups: endopeptidases and exopeptidases. Endopeptidases cleave peptide links within the polypeptide chain whereas exopeptidases cleave amino acid residues at the extremities of the polypeptide. Peptidases consist of dipeptidases, tripeptidases, carboxypeptidases and aminopeptidases. Di and tripeptidases are responsible for the cleavage of smaller peptide chains. Carboxypeptidases liberate an amino acid residue at the C-terminal end of the peptide (Barrett, 1994; Gonzales and Robert-Baudouy, 1996; Muntz, 1996), whereas aminopeptidases are exopeptidases that catalyse the cleavage of amino acid residues at the N-terminal position of peptides and proteins (Rawlings and Barrett, 1993).

Bacterial aminopeptidases may be sub-divided into three main catalytic groups: metallo-aminopeptidases, cysteine aminopeptidases and serine aminopeptidases. The metallo-aminopeptidases constitute the largest group of aminopeptidases comprising two thirds of these enzymes. These are enzymes whose activity is inhibited by metal chelating compounds e.g. EDTA and hydroxyquinoline (Rawlings, 1998; Holz, 2002). In spite of the fact that the identification of the metal ion participating in catalysis is not always possible, it was found that the most numerous group are zinc dependent aminopeptidases (Gonzales and Robert-Baudouy, 1996). For example, peptidase N found in *E. coli* is a metallo-aminopeptidase containing at least one zinc ion per monomer. It is responsible
exclusively for the hydrolysis of certain chromogenic peptidase substrates within bacteria (e.g. ala β-naphthylamide) (Geiss et al., 1985; Niven et al., 1995; Klein and Henrich, 1998). A group of enzymes containing in the active site of each subunit two Co^{++} ions has also been distinguished amongst the metal dependent aminopeptidases. Such mechanism of catalysis was described for methionine aminopeptidase from E. coli (Roderick and Matthews, 1993; Lowther and Matthews, 2000). Another group within the metallo-aminopeptidases are those which possess two zinc ions per monomer, for example peptidase A of E. coli. Peptidase A is a broad spectrum aminopeptidase that is able to remove many N-terminal amino acids from peptides in which the amino acid is adjacent to the N terminus (Stirling et al., 1989; Burley et al., 1992; Taylor, 1993). Cysteine and serine aminopeptidases require a highly reactive cysteine or serine residue for catalysis. These aminopeptidases are less common than metallo-aminopeptidases. Enzyme reactions begin with nucleophilic attack of the sulphur of the sulphydryl group (cysteine aminopeptidases) or the oxygen of the hydroxyl group (serine aminopeptidases) (Rawlings and Barrett, 1994; Mistou and Gripon, 1998; de Palencia et al., 2000; Komeda et al., 2003).

The aim of this chapter was to investigate the mechanisms of peptide transport and utilization in C. jejuni by assessing peptide utilization in the wild type strain and strains deficient in putative peptide transporters and peptidases. An alternative approach used in this study was to isolate and characterise randomly generated transposon mutants in these systems using toxic peptide derivatives as a screening technology.
5.2 Results

5.2.1 Utilization of peptides by *Campylobacter jejuni* during growth

The results in Chapter 4 demonstrated that *C. jejuni* was able to use serine and aspartate as sole carbon sources. Thus, to determine whether this pathogen could utilize peptides, its growth was assessed in GMEM medium when a variety of peptides rich in these amino acids (Table 5.1) were included as carbon sources. Cells were incubated for 5 days and the final OD$_{600nm}$ assessed. As expected *C. jejuni* could satisfy its carbon requirements by the utilization of serine and aspartate. Peptides containing either two or four aspartate supported some growth but this was less than when aspartate was provided alone. Serine was able to support more growth of *C. jejuni* than aspartate (Chapter 3) and when it was present in peptides containing four or seven residues, more growth ensued than when peptides containing aspartate alone were provided. It would appear then that *C. jejuni* can utilize peptides containing both serine and aspartate as carbon sources.

Table 5.1: Growth of *C. jejuni* in GMEM with amino acids and short peptides.

<table>
<thead>
<tr>
<th>Medium</th>
<th>OD 600nm *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.007</td>
</tr>
<tr>
<td>L-serine 5mM</td>
<td>0.676</td>
</tr>
<tr>
<td>Aspartate 5mM</td>
<td>0.158</td>
</tr>
<tr>
<td>Asp-Asp 5mM</td>
<td>0.069</td>
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<tr>
<td>Asp-Asp-Asp-Asp 5mM</td>
<td>0.064</td>
</tr>
<tr>
<td>Arg-Gly-Asp-Ser 5mM</td>
<td>0.172</td>
</tr>
<tr>
<td>Arg-Gly-Asp-Ser-Pro-Ala-Ser 5mM</td>
<td>0.203</td>
</tr>
</tbody>
</table>

* Final OD$_{600}$ nm measured after 5 days growth.
Professor Duncan Maskell’s group (University of Cambridge) had constructed defined mutants in Cj1580c-1584c, a putative ABC transport system for peptides, and Cj0653c, a putative aminopeptidase. These mutants were kindly provided by this group and since both systems were possible candidates for peptide utilization in \textit{C. jejuni} they were assessed, in comparison with the wild type for, growth on the peptides that contained serine (Table 5.2).

Table 5.2: The use of amino acids and serine containing peptides by \textit{C. jejuni} and mutants deficient in Cj1580c-1584c and Cj0653c.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Control***</th>
<th>L-Serine 5mM</th>
<th>Arg-Gly-Asp-Ser 5mM</th>
<th>Arg-Gly-Asp-Ser-Pro-Ala-Ser 5mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*ZT</td>
<td>**EE</td>
<td>*ZT</td>
<td>**EE</td>
</tr>
<tr>
<td>\textit{C. jejuni} 11168</td>
<td>0.012</td>
<td>0.003</td>
<td>0.018</td>
<td>0.544</td>
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</tbody>
</table>
| \(
\Delta
\text{Cj}
1580\text{C-}1584\text{c::kan}^R
\) | 0.013 | 0.004 | 0.018 | 0.268 | 0.012 | 0.095 | 0.014 | 0.081 |
| \text{Cj} 0653c::Tn,cm^R | 0.015 | 0.004 | 0.017 | 0.348 | 0.017 | 0.208 | 0.013 | 0.133 |

* \(\text{OD}_{600\text{nm}}\) measured at time zero
** \(\text{OD}_{600\text{nm}}\) measured at the end of experiment (5 days)
*** Control GMEM media with no added carbon source.
When serine was used as a carbon source the final OD\textsubscript{600nm} values for the Cj1580c-1584c and Cj0653c mutants were 49% and 64% of that measured for the wild type. Thus for reasons unknown the two mutant strains have a general growth defect. For the wild type strain the OD measured in the presence of Arg-Gly-Asp-Ser was 35% of that measured when L-serine was provided. As the differential in final OD was 35%, and 60%, for the Cj1580c-1584c and Cj0653c mutants, compared to that seen with serine, neither system appears to be important for uptake and utilization of the peptides used here. A similar pattern of results was seen when Arg-Gly-Asp-Ser-Pro-Ala-Ser was used.

5.2.2 The effect of toxic amino acid derivatives on the growth of C. jejuni

Since the experiments with the defined mutants above did not reveal potential systems involved in peptide utilization, an approach using random mutagenesis and a selection system was attempted here. Professor Maskell (University of Cambridge) provided a mutant library of C. jejuni, made using the pFalcon plasmid containing the solo transposon (Hendrixson et al., 2001) and containing 5000 individual mutants. The selection system was based on the toxic peptides Triornithine (H-Orn-Orn-Orn-H) (De Felice et al., 1973; Tsuhako et al., 1998), Bialophos (Bayer et al., 1972; Calanduoni and Villafranca, 1986) and L-Ala-AEP (Allen et al., 1978) which have been used previously to select for mutants deficient in peptide transport mechanisms (Gibson et al., 1984; Higgins and Gibson, 1986; Abouhamad et al., 1991). Triornithine exerts it activity by inhibiting protein synthesis (Gilvarg and Levin, 1972), while Bialaphos is a tripeptide antibiotic consisting of an analogue of L-glutamic acid (PPT) and two L-Alcemic...
residues. Upon removal of these residues, by peptidases, PPT is a potent inhibitor of glutamine synthesis. Bialaphos is taken up by both the oligopeptide and dipeptide system and organisms that grow in the presence of Bialaphos lack the dpp/opp transport system (Solomon et al., 2003).

The first step in this experiment was to develop a suitable screen for peptide transport deficient mutants. The toxicity of the peptide derivatives was initially assessed at concentrations of 200 μM, 250 μM, 300 μM and 400 μM using MHA in a standard disc diffusion assay. However, as no zones of inhibition were seen (data not shown), and as this was most likely due to the presence of competing natural peptides in the complex MHA interfering with toxic peptide uptake, the experiment was repeated in the defined GMEM media. Figure 5.1) shows that L-Ala-AEP (200 μM) was able to produce clear zone on GMEM media whilst there were no zones found around H-Orn-Orn-Orn-H (200 μM) and Bialophos (200 μM).
5.2.3 The spectrum of toxicity of L-Ala-AEP against different strains of *Campylobacter*

Of the toxic peptides tested only L-Ala-AEP gave rise to a zone of inhibition and the use of this compound was explored further to determine the most appropriate concentration for use and whether the activity of this compound extended to other *Campylobacter* strains.

Strains were grown in GMEM (FeSO₄ 50 μM, HEPES 25mM and sodium pyruvate 20mM) and growth assessed in the presence or absence of L-Ala-AEP by measuring...
OD$_{600\text{nm}}$ after five days growth (Table 5.3). This compound was toxic to all three of the strains of *C. jejuni* assessed whilst three out of the four strains of *C. coli* tested were resistant. This may reflect differences in peptide transport between different strains.

Table 5.3: Growth of different strains of *Campylobacter* in GMEM with and without L-Ala-AEP (100 μM).

<table>
<thead>
<tr>
<th><em>Campylobacter</em> strains</th>
<th>GMEM without L-ala-AEP</th>
<th>GMEM with L-ala-AEP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OD$_{600\text{nm}}$</td>
<td></td>
</tr>
<tr>
<td><em>C. jejuni</em> 11168</td>
<td>0.344</td>
<td>0.004</td>
</tr>
<tr>
<td><em>C. jejuni</em> 11828</td>
<td>0.201</td>
<td>0.005</td>
</tr>
<tr>
<td><em>C. jejuni</em> 11951*</td>
<td>0.227</td>
<td>0.001</td>
</tr>
<tr>
<td><em>C. coli</em> UA585</td>
<td>0.515</td>
<td>0.439</td>
</tr>
<tr>
<td><em>C. coli</em> 122110</td>
<td>0.322</td>
<td>0.294</td>
</tr>
<tr>
<td><em>C. coli</em> 11366</td>
<td>0.310</td>
<td>0.004</td>
</tr>
<tr>
<td><em>C. coli</em> 11437</td>
<td>0.379</td>
<td>0.332</td>
</tr>
</tbody>
</table>

* This strain was also grown in the presence of L-serine and glutamine as these are required for growth of this strain.
Chapter 5  The mechanisms of peptide transport and utilisation in Campylobacter

The growth profiles of a resistant stain (C. coli UA585) and a strain sensitive to L-Ala-AEP are shown in Figure 5.2.

![Growth curve of C. jejuni 11168 and C. coli UA585 in GMEM with FeSCL (50 pM), HEPES (25mM) and sodium pyruvate (20mM), supplemented with and without 300 pM L-Ala-AEP. The medium was inoculated at time zero, with C. jejuni 11168 / C. coli UA585 and cultures grown at 37 °C under microaerobic conditions with shaking. This experiment was performed three times on three independent occasions and similar reproducible results obtained.](image)

Figure 5.2: Growth curve of C. jejuni 11168 and C. coli UA585 in GMEM with FeSO₄ (50 μM), HEPES (25mM) and sodium pyruvate (20mM), supplemented with and without 300 μM L-Ala-AEP. The medium was inoculated at time zero, with C. jejuni 11168 / C. coli UA585 and cultures grown at 37 °C under microaerobic conditions with shaking. This experiment was performed three times on three independent occasions and similar reproducible results obtained.

The disc diffusion assay was also used to establish whether the defined mutants provided by Cambridge group were resistant to L-Ala-AEP. As seen in Table 5.4, the Cj1580c-1584c mutant showed increased resistance to this agent whilst the Cj0653c mutant was resistant and whilst these systems do not appear to be important for the uptake of the peptides examined in Table 5.1 and Table 5.2, they appear to play important roles in L-Ala-AEP uptake and toxicity.
Table 5.4: A disc diffusion assay to assess the toxicity of L-Ala-AEP against defined *C. jejuni* mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Disk load (μmol)</th>
<th>Inhibition zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>11168</td>
<td>100 μM</td>
<td>45 mm</td>
</tr>
<tr>
<td>Δ Cj 1580C-1584c::kanR</td>
<td>100 μM</td>
<td>21 mm</td>
</tr>
<tr>
<td>Cj 0653c::Tn,cmR</td>
<td>100 μM</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

Since the intention of this experiment was to plate a mutant library of *C. jejuni* onto agar plates containing L-Ala-AEP and then to select for resistant mutants, the inhibitory effects of different concentrations of this agent were assessed in solid GMEM. Cell suspensions containing 1 x 10^7 CFU ml^-1 were diluted to 10^-1, 10^-2 and 10^-3 in MRD were plated onto GMEM containing 200 μM, 250 μM or 300 μM L-Ala-AEP and incubated for 5 days. When this experiment was carried out with the Cj0653c (resistant L-Ala-AEP) colonies grew at all dilutions confirming that these concentrations could be used to select for resistant strains. Conversely, colonies of NCTC 11168 failed to grow on GMEM containing 250 μM and 300 μM confirming that a sensitive strain would not grow at these concentrations. At 200 μM five resistant colonies arose, which were presumably naturally occurring mutants. To confirm that these were indeed resistant mutants, one was taken and grown in liquid media containing L-Ala-AEP. As can be seen in Figure 5.3, the mutant was resistant to this agent whilst the wild type was sensitive. When the transposon solo library was plated onto GMEM containing either
250 μM or 300 μM L-Ala-AEP also containing 10μg ml⁻¹ chloramphenicol to maintain transposon selection, no resistant colonies were detected amongst the 5000 mutants.

Figure 5.3: Comparison of the growth of *C. jejuni* 11168 wild type and a *C. jejuni* mutant resistant to Ala-AEP in GMEM with FeSO₄ (50 μM), HEPES (25mM) and sodium pyruvate (20mM), supplemented with and without 200 μM L-Ala-AEP. The medium was inoculated at time zero and cultures grown at 37 °C under microaerobic conditions with shaking. This experiment was performed three times on three independent occasions and similar reproducible result obtained.
5.3 Discussion

Using the defined medium developed earlier in this study, the ability of *C. jejuni* to use a range of peptides containing two, four and seven amino acids was assessed. When provided as a sole carbon source, peptide containing 2 or 4 aspartate residues allowed some growth but this was less than was seen when a similar concentration of aspartate was provided. Peptides of 4 and 7 amino acids, which also contained serine, stimulated growth in a more dramatic fashion which might reflect the fact that serine in general supports better growth of *C. jejuni* (Chapter 3). In any case the research in this chapter demonstrates for the first time that this pathogen is able to use peptides as sources of carbon. The mechanisms via which peptides are transported are not yet known but these must be present in *C. jejuni*. In a recent study (Weinberg and Maier, 2007) carried out using *H. pylori*, which is related to *C. jejuni*, analysis of genome sequence revealed putative ABC-type transporter systems for both dipeptides (DppABCDF) and oligopeptides (OppABCD). A mutant deficient in the dipeptide system lacked the ability to use certain dipeptides, hexapeptides, and nonapeptides but retained some ability to grow with other dipeptides, tripeptides, and tetrapeptides. In addition an oppB mutant strain showed a wild-type phenotype for growth with longer peptides (hexa- and nonapeptides) but had decreased ability to utilize di-, tri-, and tetrapeptides. An as yet unidentified peptide transport system(s) in *H. pylori* was proposed to be responsible for the residual transport in opp, dpp double mutants. In *C. jejuni* genes Cj1580c-1584c are predicted to encode a putative ABC type peptide transporter with homology to Dpp in *E. coli* (Parkhill et al., 2000). Cj1580c is annotated as an ATP-binding protein component
of peptide ABC-transport system, while Cj1581c is also annotated as a potential ATP-binding part of peptide ABC-transport system protein. Cj1582c is annotated as the probable peptide ABC-transport system inner membrane permease protein, as is Cj1583c. Cj1584c is annotated as probable peptide ABC-transport system periplasmic peptide-binding protein and has similarity to oligopeptide-binding protein AppA (Parkhill et al., 2000; CampyDB, 2008).

To determine whether Cj1580c-1584c was involved in uptake of peptides, a mutant deficient in this system was acquired from Professor Duncan Maskell’s group at the University of Cambridge. The same group also kindly provided a mutant deficient in Cj0653c, a putative amino peptidase. Since both components were possible candidates for peptide utilization in C. jejuni they were assessed, in comparison with the wild type, for growth on the peptides containing serine. However, relative to the wild type growth of neither mutant was diminished, when peptides of four and seven amino acids, and also containing serine were included in the defined growth medium. Thus, Cj1580c-1584c is not involved in the transport of the peptides assessed here but it might be involved in the transport of other peptides with different characteristics to those tested. Given the vague specificities of the oligopeptide uptake systems in H. pylori (Weinberg and Maier, 2007) this seems probable.

This chapter also investigated the toxicity of various toxic peptide analogues against C. jejuni as a prelude to using these as selective agents for the isolation of mutants deficient in peptide uptake. Interestingly, C. jejuni unlike Salmonella and E. coli (De Felice et al.,
1973; Morley et al., 1983; Payne, 1983; Higgins and Gibson, 1986) was found to be resistant H-Orn-Orn-Orn-H and Bialophos and this might be due to the different rates of transport of the substrates and to different hydrolysis rates of enzymes within the organism. This is an interesting observation which could potentially lead to the use of these two toxic peptide derivatives as selective agents in C. jejuni selective media. C. jejuni was found however to be susceptible to L-Ala-AEP. When different strains of C. jejuni and C. coli were assessed, for L-Ala-AEP sensitivity, three out of three strains of C. jejuni tested were sensitive and three of four stains of C. coli tested were resistant. This most likely reflects the existence of different mechanisms of peptide uptake or degradation in strains of Campylobacter. Early studies by Allen et al., (1979) showed that resistance to L-Ala-AEP was very low (0-5%) in Gram negative organisms, and thus the finding that most C. coli strains were resistant is unusual. For an organism to be susceptible to this agent it must posses an uptake system for L Ala-AEP and an alanine aminopeptidase, which cleaves the compound and allows the release of the cell wall inhibitor AEP, and thus resistance in the C. coli strains may be due to deficiencies in either of these mechanisms. Similarly L Ala-AEP had modest antibacterial activity against Proteus, Pseudomonas spp. and streptococci (with the exception of Streptococcus faecalis) (Allen et al., 1978; Allen et al., 1979; Atherton et al., 1979). In the genus Streptococcus resistance is attributed to the lack of permeases capable of transporting dipeptides and consequently these organisms take up almost no L-Ala-AEP (Atherton et al., 1980).
The finding in this chapter that a Cj1580c-1584c mutant showed elevated resistance to L-Ala-AEP compound, compared to the parental strain, demonstrates that this putative ABC-type peptide transporter plays a role in L-Ala-AEP transport but also that other systems must also be involved and that these account for the residual sensitivity. In addition, since a mutant deficient in Cj0653c, a putative amino peptide was resistant to L-Ala-AEP this enzyme must possess the only alanine aminopeptidase activity in C. jejuni.

Previous work has shown that L-Ala-AEP is taken up by the tripeptide permease system in Salmonella and therefore Tpp deficient mutants arise when organisms normally sensitive to the effects of this agent grow in the presence of L-Ala-AEP (Gibson et al., 1984). Here a screen using L-Ala-AEP and solid GMEM was developed that could clearly differentiate between sensitive (wild type) and resistant (Cj0653c) strains of C. jejuni. However, when a transposon library containing 5000 different mutants was applied to the plates no resistant colonies were detected. Since the screen was demonstrated to be effective, this suggests that the coverage of mutated genes in the transposon library was not sufficient to ensure all genes likely to be involved in peptide transport or processing were inactivated in the library. Nevertheless, a naturally occurring L-Ala-AEP mutant was isolated and future characterization of this strain might provide insight into peptide utilization in C. jejuni.
Chapter 6

Substrate accelerated death in *Campylobacter jejuni*

6.1 Introduction

Although, growth is not possible in the environment, the survival of campylobacters here plays a vital role in maintaining their infection cycle. The survival of *C. jejuni* in surface waters is dependent on a number of factors such as temperature, the presence of oxygen and the availability of nutrients. For example, the rate of loss of viability in river water, a microcosm containing nutrients is markedly less than that observed in de-ionized water (Hazeleger *et al.*, 1998; Thomas *et al.*, 1999). The duration of survival and response to water nutrient composition in *Campylobacter* was less than *E. coli* (Cook and Bolster, 2007). Whilst survival is poor at higher temperatures (37 °C), it is optimal at low temperatures (5-15 °C), which do not promote growth and which correlate to some environmental temperatures (Thomas *et al.*, 1999; Mihaljevic *et al.*, 2007). Survival of thermophilic *Campylobacter* was better at 4 °C than at 25 °C (Tatchou-Nyamsi-Konig *et al.*, 2007). Garenaux *et al.*, (2008), recently found that *C. jejuni* was more resistant at 4 °C to oxidative stress than at 42 °C which is its optimal growth temperature. Oxygen consumption, catalase activity, ATP generation and protein synthesis still occur at
temperatures as low as 4°C, indicating that vital cellular processes were still functioning (Alter and Scherer, 2006).

When exposed to environmental stress certain bacteria may enter a viable non-culturable (VNC) state. This concept, of a bacterium that remains infectious but that can no longer be cultured by conventional means, was first proposed by Colwell following a study on the survival of Salmonella in aquatic systems (Roszak et al., 1984). Such bacteria may retain metabolic activity yet are unable, under the prevailing environmental conditions, of undergoing the cellular division required for growth. Furthermore, conversion from the VNC form has shown to be reversible with the advent of improved environmental conditions (McKay, 1992). A VNC form of C. jejuni was first reported by Rollins and Colwell (Rollins and Colwell, 1986) and since then there has been continuous debate as to whether a VNC form for Campylobacter actually exists but given the fastidious nature of Campylobacter, the loss of culturability in this organism is easy to induce. For example, cultures rapidly lose viability, as assessed by plate counting, following exposure to oxygen, changes in temperature, and starvation (Park, 2002). Cools et al., (2003) found that starved C. jejuni lost culturability more quickly on selective media, and that resuscitation was also influenced by the media type. Recovery or reversion of the VNC form has been reported by a number of groups but these studies have often generated controversy. Accordingly, the recovery of VNC forms, which are not detectable by conventional culture techniques, has been reported in a number of different animal models (Saha et al., 1991; Jones et al., 1991), and more recently in embryonated eggs (Cappelier et al., 1999). In contrast, a number of studies have failed to induce the
recovery of VNC cells of *C. jejuni* in animal models (Beumer et al., 1992; Medema et al., 1992) and these results have cast doubts on the existence of a VNC form and the role that it plays in the environmental transmission of campylobacters. The decline in viability and loss of culturability in campylobacters, that occurs following exposure to certain unfavorable environments, is often associated with a change in cell morphology from a spiral rod-like form into a coccoid form (Rollins and Colwell, 1986). Since the rate of appearance of the coccoid form often parallels the loss in culturability, much research has focused on the possibility that the coccoid form is in fact the dormant VNC stage of *C. jejuni*.

In Chapter 3 of this thesis mucin, serine, pyruvate, and aspartate were identified as sole carbon sources that could be used by *C. jejuni* for growth. Whilst many studies have examined *Campylobacter* survival none has investigated how the availability of carbon source influences survival. The aim of this chapter, therefore, was to characterise the influence of the above carbon sources on the survival of *C. jejuni* under aerobic conditions.
Chapter 6  
Substrate accelerated death in Campylobacter jejuni

6.2 Results

6.2.1 Survival of Campylobacter jejuni as affected by the presence of mucin

Preliminary work by Karen Elvers (University of Surrey) had shown that the survival of C. jejuni, following exposure to air, was highest in MRD when this was compared to other low nutrient environments (GMEM, water, PBS) and this medium was, therefore, chosen for the survival studies performed here. Since mucin was readily utilized for C. jejuni growth, survival of C. jejuni in the presence or absence of this carbon source was assessed. The presence of mucin clearly affected the survival of the cells in a concentration dependent manner in that with 0.5% mucin there was a small increase in survival (less than 0.5 logs) whilst in the presence of 1% mucin survival throughout the experiment was generally 1-log higher than in its absence (Figure 6.1). From time zero to 8 hours there was even an increase in cell numbers from 6.2 logs to 6.7 logs, in the presence of 1% mucin.

Clearly mucin has a protective effect and to determine whether it could protect cells which had already survived in its absence, the survival experiment was repeated but this time mucin was only added 10 hours after the survival experiment had begun (Figure 6.2). The culturability of surviving cells was clearly enhanced by the addition of 1% mucin, which when added resulted in an increase in cell count from 5.7 logs to 6 logs over a 2 hour period, when viability in the absence of this carbon source declined by 1-log. The influence of mucin was concentration dependent. In the presence of 0.5% mucin
there was no stimulation of viability and the protective effect was less pronounced and no measurable effect when 0.1% mucin was used.

Figure 6.1: The survival of *Campylobacter jejuni* in MRD to which different concentrations of mucin had been added. Flasks were inoculated at time zero with *C. jejuni* NCTC 11168 and cultures incubated at 25°C under aerobic conditions with shaking 155 RPM.
Figure 6.2: The survival of *C. jejuni* under aerobic conditions and the effect of mucin addition 10 hours after incubation. Cells of *C. jejuni* NCTC 11168 were inoculated into MRD and time zero and incubated at 25°C under aerobic conditions with shaking.

### 6.2.2 The effect of the addition of individual carbon sources to surviving cells of *C. jejuni*

Mucin is a complex material containing different amino acids and sugars. This study next sought to determine whether the addition of individual carbon sources affected *C. jejuni* during survival. In the first instance, the survival experiment was repeated by adding serine and pyruvate to cells following 10 hours of survival (Figure 6.3) as these were readily used as carbon sources by *C. jejuni*. 

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Figure 6.3: The effect of the addition of L-serine and sodium pyruvate on the survival of *C. jejuni*. MRD was inoculated at time zero and cells incubated in MRD media at 25 °C under aerobic conditions. After 10 hours L-serine (20 mM) and sodium pyruvate (20 mM) were added and the experiment continued.

The addition of either serine or pyruvate brought about a dramatic and immediate reduction in viability and just 3 hours after the addition viability had dropped by over 3-logs. Thereafter, whilst the control population contained 2.5 x 10 CFU ml⁻¹, after 20 hours survival, in the flasks to which pyruvate and L-serine had been added no viable bacteria were recovered. In contrast, in the control population reached undetectable levels only after 50 hours incubation.

This experiment was repeated, but with the additions made after 20 hours of survival, and also by including aspartate, which is also used by *C. jejuni* as a carbon source (Figure 6.4). The addition of all three carbon sources brought about a rapid decline in viability
and 3 hours after addition viability had declined by 2-logs. Thereafter cell numbers declined most in the flask to which aspartate had been added and least in that to which serine had been added but in all cases viability was less than that measured in the control with no carbon source addition.

To determine whether cell death resulted from the addition of amino acids and carboxylic acids \textit{per se}, or whether it was dependent on the use of them as carbon sources, a survival experiment was carried in which pyruvate and threonine (an amino acid that cannot be used as a carbon source) were added to cells either 10 or 20 hours after that start of the survival experiment (Figure 6.5). The addition of pyruvate brought about the same decline in viability as had been observed previously at both addition points. However, the addition of threonine at little effect on viability suggesting that it is the use of the amino acids and carboxylic acids as carbon sources that gives rise to the cell killing.

Pyruvate which induced substrate accelerated death is metabolised and therefore may induce pH changes in the media which bring cell death therefore pH was measured (Table 6.1). The addition of pyruvate at both time points results in an increase in media alkalinity but in both cases this was under 0.5 pH units and is an unlikely cause of enhanced cell death.
Figure 6.4: The effect of the addition of L-serine, sodium pyruvate, and aspartate on the survival of *C. jejuni*. MRD was inoculated at time zero and cells incubated in MRD media at 25 °C under aerobic conditions. After 20 hours L-serine (20 mM), sodium pyruvate (20 mM), or aspartate (20 mM), were added and the experiment continued.

Figure 6.5: The effect of the addition of sodium pyruvate and threonine on the survival of *C. jejuni*. MRD was inoculated at time zero and cells incubated in MRD media at 25 °C under aerobic conditions. After 10 and 20 hours, sodium pyruvate (20 mM) or threonine (20 mM) and were added and the experiment continued.
Table 6.1: pH changes of the media when pyruvate and threonine were added at 10 and 20 hours.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time(h)</th>
<th>C. jejuni 11168 control (no addition)</th>
<th>C. jejuni 11168 with pyruvate (10h)</th>
<th>C. jejuni 11168 with pyruvate (20h)</th>
<th>C. jejuni 11168 with Threonine (10h)</th>
<th>C. jejuni 11168 with Threonine (20h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
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<tr>
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<td>7.14</td>
<td>7.15</td>
<td>6.9</td>
<td>6.94</td>
</tr>
</tbody>
</table>
In this chapter the affect of the addition and availability of carbon sources on the survival of *C. jejuni* was investigated.

Mucin was clearly protective and cells survived better in its continual presence. Moreover, when it was added to cells that had survived in its absence the recovery of viable cells was increased. This effect was related to the concentration of mucin with 0.1% having no effect and 1% mucin, the highest concentration used, stimulating and increase in cell count of at least 10-fold. It is possible that by attaching to mucin in the medium, *C. jejuni* may form cell clumps and as this might protect the cells from the harmful effects of the oxygen dissolved in the surrounding liquid (Jiang and Doyle, 2000) it might account for the enhanced viability observed. However, the formation of clumps would initially result in an apparent decline in cell number, when an increase in was actually observed. This mechanism is thus unlikely to explain the protective effect of mucin. It is possible that the increased viscosity provided by mucin in the supplemented MRD may also create a more favourable microaerobic environment for *C. jejuni* survival as this would inhibit oxygen diffusion (Cellini *et al.*, 1994; Federighi *et al.*, 1998). Similarly the presence 0.2 % mucin in recovery media was shown to enhance recovery of *C. jejuni* in liquid media (Bovill and Mackey, 1997).
When carbon sources were added as single and defined compounds the effect on viability was equally striking but in the opposite direction as that seen with mucin, in that the addition of pyruvate, serine, and aspartate brought about a rapid decline in viability. This phenomenon clearly required that the additive be a usable carbon source since when threonine, which cannot be used as a sole carbon source, was added no decrease in viability was measured. Pyruvate has previously been shown enhance the growth and aerotolerance of *Campylobacter* spp. by neutralizing the toxic effect of oxygen, which contradicts findings found in this investigation (George *et al.*, 1978). Here, however, the addition of pyruvate was unbalanced and other growth promoting agents were not present and this may bring about the opposite effect. Addition of pyruvate also results in a decrease in the concentration of hydrogen peroxide, which enables *C. jejuni* to grow aerobically (Verhoeff-Bakkenes *et al.*, 2008). However, this protecting effect of pyruvate might be attributed to a switch in metabolism from aerobic respiration to fermentation when pyruvate is added, as pyruvate is used during fermentation rather than oxygen and no toxic reactive oxygen intermediates (ROI) are formed (Mendz *et al.*, 1997; Verhoeff-Bakkenes *et al.*, 2008).

When starved cells are re-supplied with nutrition, there often are problems in that the cell may not survive or at least may not return to active growth immediately due to a number of metabolic imbalances (Koch, 1997). The effect seen in this chapter may be due to one such phenomenon called substrate accelerated (activated) death (Postgate and Hunter, 1964; Calcott and Calvert, 1981). Substrate accelerated death is a phenomenon whereby death ensues at a much higher rate when a growth-limiting substrate is reintroduced to
starved bacteria (Postgate and Hunter, 1963). In *E. coli* a related affect has been observed in that when cells are taken from a lactose limited chemostat and plated onto minimal media containing lactose 80 to 98% of the cells die (Dykhuizen and Hartlactose, 1978). This form of cell suicide is thought to be caused the rapid uptake of sugars by the lactose permease and the subsequent disruption of membrane function. Similarly, maltose pulses to a maltose-limited chemostat culture of *Saccharomyces cerevisiae* resulted in substrate-accelerated death and this most likely occurred due to uncontrolled uptake of maltose into the cell, resulting in an osmotic burst (Postma et al., 1990). In addition glycerol-limited cells of *Aerobacter aerogenes* showed glycerol-accelerated death (metabolites of glycerol, e.g., pyruvate, also accelerated death) (Postgate and Hunter, 1963). Strange and Dark (1966) could not confirm substrate-accelerated death with ammonium or phosphate-limited populations but suggested that the toxic product of substrate utilization was responsible for the accelerated death. These findings may provide an explanation of the mechanism of substrate-accelerated death that metabolism of carbon substrates in absence of other nutrients.
Chapter 7

Use of the defined media to establish iron acquisition and transport of compatible solutes in C. jejuni

7.1 Introduction

To successfully grow in the gastrointestinal tract or to disseminate to extra-intestinal sites, C. jejuni must have mechanisms for acquiring iron from its hosts (Bullen, 1981; Finkelstein et al., 1983). Iron availability has also been shown to be a key signal for pathogens to sense that they have invaded the host (Litwin and Calderwood, 1993; Ratledge and Dover, 2000). On the other hand, uncontrolled iron uptake causes iron toxicity and oxidative stress, leading to cessation of growth (Touati, 2000), because iron is also capable of generating toxic oxygen radicals in the presence of oxygen. Such radicals can damage DNA, proteins and membranes. Consequently iron homeostasis is of critical importance to living organisms and this is achieved by tightly controlling the uptake, metabolism, and storage of iron. The expression of iron-regulated systems in Gram-negative bacteria is generally controlled by the Fur (ferric uptake regulator) protein, which represses the transcription of iron-regulated promoters by using Fe$^{2+}$ as a cofactor (Stojiljkovic et al., 1994; Hassett et al., 1996; Hantke, 2001). In C. jejuni, Fur
was found to regulate the expression of several proposed iron-uptake systems, including Ceu, CfrA, ChuA and p19 (van Vliet et al., 1998; Holmes et al., 2005), but unlike the fur gene of many other bacteria, C. jejuni fur is not autoregulated (van Vliet et al., 2000). It has been suggested that Fur binds to specific DNA sequences overlapping Fur-regulated promotors when the intracellular Fe$^{2+}$ concentration is high enough to allow the formation of a complex consisting of Fur dimer and Fe$^{2+}$ (van Vliet et al., 2000; Holmes et al., 2005).

Since the genome of strain NCTC11168 was sequenced (Parkhill et al., 2000), a number of putative iron acquisition genes have since been identified; for example the ABC Fe$^{2+}$ iron transporter, FeoB. In Legionella, FeoB is important for extracellular and intracellular growth especially in iron-limited environments (Robey and Cianciotto, 2002). It also plays a role for Salmonella in intracellular survival and replication in macrophages (Boyer et al., 2002). Moreover, FeoB mutants in Helicobacter are unable to colonise the gastric mucosa of mice (Velayudhan et al., 2000). However, characterisation of the putative FeoB in C. jejuni has suggested that it was not involved in iron uptake (Raphael and Joens, 2003).

Campylobacter species do not synthesize siderophores but they are able to use high affinity iron chelators, ferrichrome and enterochelin produced by other organisms or from other sources (Pickett et al., 1992; van Vliet et al., 2002; Palyada et al., 2004). Other enteric pathogens such as E.coli have been shown to be able to utilize host derived catechol compounds such as epinephrine and adrenaline to obtain iron during infection.
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Iron acquisition and transport of compatible solutes in C. jejuni

(Lyte and Ernst, 1992; Bansal et al., 2007; Freestone et al., 2002). *Listeria monocytogenes*, another food borne pathogen has been shown to acquire iron from plant derived compounds such as rutin that may be present in the diet of the host or in the environment (Simon et al., 1995). Because the minimal media developed here can be readily made to be iron limiting, one of the aims of this chapter was to examine its use for determining the utilization of iron sources by *C. jejuni*.

The internal osmotic pressure in bacterial cells is higher than that of the surrounding medium and these results in a pressure exerted outwards on the cell wall, called the turgor pressure, which is thought to provide the mechanical force necessary for cell elongation (Csonka, 1989). Consequently, bacterial cells must be able to maintain turgor despite variations in the osmotic pressure of the surrounding medium. *C. jejuni* is more sensitive to osmotic stress than other important foodborne pathogens (Doyle and Roman, 1982a; Park, 2002; Kelana and Griffiths, 2003). Consequently, *C. jejuni* is quite sensitive to sodium chloride and it is not able to survive in the presence of high sodium chloride concentrations (Birk et al., 2004; Hamedy et al., 2005). The optimal sodium chloride concentration for recovering or enumerating *C. jejuni* is 0.5% NaCl (Abram and Potter, 1984). In addition, whereas strains multiplied in media made which chicken juice supplemented with 0% and 1% sodium chloride, the bacterial counts did not change significantly when 2% sodium chloride was added. Growth did not occur and cell number gradually decreased in chicken juice containing 3% sodium chloride (Hamedy et al., 2005). In contrast, *Salmonella* will grow in sodium chloride concentrations of 4.5%
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and 10%, respectively (ICMSF, 1996; Park, 2002). A variety of organisms accumulate the so-called osmoprotectants betaine and proline to prevent dehydration when placed under the stress of high-osmolarity environments (Stewart and Lee, 1974; Hocking, 1988; Csonka, 1989). Betaine is mainly present in foods of plant origin (Rhodes and Hanson, 1993). Csonka (1989) observed that when betaine or proline is present in the growth medium, large amounts of the solute are transported into the cytoplasm by the osmotically regulated proU and proP gene products. Furthermore, the activity of the ProP and ProU systems is significantly increased upon exposure of cells to an osmotic upshift (Culham et al., 1993). The addition of low concentrations of betaine to the medium raises the upper limit of external osmolarity at which growth occurs (Chambers and Kunin, 1985), reduces the amount of time required for growth to resume after osmotic upshift (Sutherland et al., 1986), and increases the growth rate of osmotically stressed E. coli and S. Typhimurium (Perroud and Le Rudulier, 1985). The genome sequence of C. jejuni (Parkhill et al., 2000) suggests that whilst this organism may possess an orthologue of ProP, a low affinity transporter for proline and glycine betaine (Cairney et al., 1985b), it does not possess any previously characterised high-affinity transporters for known compatible solutes such as the ProU system for betaine transport (Stirling et al., 1989). ProU is a relatively specific, binding protein-dependent transport system with an affinity for glycine betaine (May et al., 1986; Barron et al., 1987), and is involved in the active uptake of glycine betaine and of L-proline from the medium in cells subjected to water stress and in the consequent ability of these compounds to promote growth in media of otherwise inhibitory osmolarity (Cairney et al., 1985b;
Gowrishankar, 1986; Milner et al., 1987). Also the ProU transport system is regulated by external osmolarity in order to optimize glycine betaine accumulation (Cairney et al., 1985a; May et al., 1986). Campylobacters also appear to lack the ability to synthesise compatible solutes by known pathways since they lack the osmoregulatory betaine and trehalose synthesis pathways (Lamark et al., 1991; Strom and Kaasen, 1993).

Given that the defined medium developed here lacks compatible solutes one of the aims of this chapter was to determine the use of compatible solutes by C. jejuni at elevated osmolarities.
7.2 Result

7.2.1 The ability of *C. jejuni* to use different iron containing compounds

The capability of *C. jejuni* strains to acquire iron from various iron and heme-containing compounds (Simon *et al*., 1995; Coulanges *et al*., 1998), was initially tested using plate assays (Table 7.1) and (Figure 7.1). As FeSO₄ and heamin have been shown to be utilised as sources of iron previously these were used as positive controls. *C. jejuni* cells were seeded into GMEM solid medium without added iron. Filter paper discs loaded with various iron containing compounds were then placed onto the media to determine whether these stimulated growth. Epinephrine, quercetin, DL-neorepinephrine and FeSO₄ stimulated growth, whereas heamin, rutin and caffeic acid stimulated growth but also gave rise to an inhibition growth zone close to the disks. Catechin failed to stimulate growth. Examples of this effect can be seen in Figure 7.1.

Table 7.1: Growth promoting effect of various iron containing compounds on *C. jejuni* grown on solid iron-deficient GMEM media.

<table>
<thead>
<tr>
<th>Ligand of iron complex</th>
<th>Disk load (µ mol)</th>
<th>Zone diameter (mm)</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemin</td>
<td>0.750 µ mol</td>
<td>45</td>
<td>13</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>0.750 µ mol</td>
<td>44</td>
<td>NZ</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.750 µ mol</td>
<td>40</td>
<td>9</td>
</tr>
<tr>
<td>DL-neorepinephrine</td>
<td>0.750 µ mol</td>
<td>46</td>
<td>NZ</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.750 µ mol</td>
<td>34</td>
<td>10</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.750 µ mol</td>
<td>42</td>
<td>NZ</td>
</tr>
<tr>
<td>Catechin</td>
<td>0.750 µ mol</td>
<td>0</td>
<td>NZ</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>0.750 µ mol</td>
<td>38</td>
<td>NZ</td>
</tr>
</tbody>
</table>

*NZ, No zone inhibition*
Figure 7.1: Growth of *C. jejuni* on GMEM, containing various iron sources on filter paper discs. A clear zone of inhibition is seen around the Caffeic acid (A) and Heamin disk (B) compared with control (C).

The ability of the growth of *C. jejuni* to be stimulated by the iron containing compounds was further examined by growing the strains in GMEM liquid media. In the absence of added iron growth was limited and the OD$_{600}$ nm did not rise above 0.15 (Figure 7.2 and Figure 7.3). Addition of various concentrations of the control compounds, haemin and FeSO$_4$, clearly stimulated growth to an OD$_{600}$ nm above 0.5. The addition of epinephrine, DL- neorepinephrine, caffeic acid, rutin and quercetin but not catechin stimulated *C. jejuni* growth but to a lesser extent then FeSO$_4$ and Haemin (Figure 7.4-Figure 7.9) suggesting that with the exemption of catechin *C. jejuni* can acquire iron from these compounds.
compounds. From the plate assay catechin appeared to be more growth inhibitory at higher concentrations and this compound may be toxic to *C. jejuni* at the concentration used in these experiments.

![Graph showing the effect of Haemin on growth of *C. jejuni* in GMEM.](image)

**Figure 7.2:** Effect of Haemin on growth of *C. jejuni* in GMEM. The media were inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of sodium pyruvate and HEPES (25mM).
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Figure 7.3: Effect of FeSO₄ on the growth of C. jejuni. Growth was monitored by measuring optical density at 600 nm. GMEM containing sodium pyruvate with HEPES (25mM) was inoculated at time zero, with C. jejuni NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking.

Figure 7.4: Growth of C. jejuni in GMEM with Epinephrine. The media were inoculated at time zero, with C. jejuni NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of sodium pyruvate.
Figure 7.5: Effect of DL-norepinephrine on growth of *C. jejuni*. The GMEM media were inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of sodium pyruvate.

Figure 7.6: Growth of *C. jejuni* in GMEM with Caffeic acid. The media were inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of sodium pyruvate.
Figure 7.7: Growth of *C. jejuni* in GMEM with Rutin. The media were inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of sodium pyruvate.

Figure 7.8: Growth of *C. jejuni* in GMEM with Quercetin. The media were inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of sodium pyruvate.
7.2.2 Comparison of *C. jejuni* growth at elevated osmolarities in defined and complex media

To determine whether the complex medium MHB contained compounds that could be used as osmoprotectant, that the defined medium GMEM did not possess, growth at elevated osmolarities was compared using both media. Figure 7.10 and Figure 7.11 illustrate the response of *C. jejuni* to salt concentrations ranging from 0.05 to 0.3M in both GMEM and MHB. In the presence of 0.05 M 0.1 M and 0.15 M salt, the growth of *C. jejuni* was substantially slower compared with the no salt control for both media. It is also evident that growth cannot occur in the presence of NaCl 0.2M or greater. Since growth in the two media was similar it would appear that MHB does not contain
compounds that can be used as compatible solutes by *C. jejuni*. To determine whether known compatible solutes could produce protection during growth at high osmolarity the growth of *C. jejuni* was assessed in the presence or absence of proline and glycine betaine. When proline was added in the absence of NaCl growth was stimulated resulting in an increase in OD after one day of culture and the OD reached 0.6 after 3 days. This may be linked to its use as a carbon source (Chapter 1). However, proline did not stimulate growth when it was added to GMEM containing elevated levels of NaCl (Figure 7.12).

These experiments were repeated using glycine betaine as the osmoprotectant (Figure 7.13). When added in the absence of NaCl glycine betaine had an inhibitory effect on growth and also rather than stimulating growth at high osmolarity led to an abolition of growth at 0.05 M NaCl because of the apparent toxicity of betaine. The experiments were repeated using a lower concentration (0.1 mM) (Figure 7.14) and even at this lower concentration betaine still possessed inhibitory activity and did not stimulate growth at elevated concentrations of NaCl.
Figure 7.10: Effect of NaCl on growth of *C. jejuni*. MHB medium was inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking.

Figure 7.11: Effect of NaCl on growth of *C. jejuni*. GMEM medium with HEPES containing FeSO₄ (50 μM) was inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of pyruvate.
Figure 7.12: Effect of NaCl and proline on growth of *C. jejuni*. GMEM medium with HEPES containing FeSO₄ (50 μM) was inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of pyruvate.

Figure 7.13: Effect of NaCl and Betaine (1 mM) on growth of *C. jejuni*. GMEM medium with HEPES containing FeSO₄ (50 μM) was inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of pyruvate.
Figure 7.14: Effect of NaCl and Betaine (0.1 mM) on growth of *C. jejuni*. GMEM medium with HEPES containing FeSO₄ (50 μM) was inoculated at time zero, with *C. jejuni* NCTC11168 and cultures grown at 37 °C in microaerobic conditions with shaking in presence of pyruvate.
7.3 Discussion

In the present chapter, the ability of different compounds to promote the growth of *C. jejuni* in iron-restricted medium was investigated. The findings have confirmed that haemin is capable of stimulating *C. jejuni* growth under iron-restricted conditions. (Figure 7.2) Ridley *et al.*, (2006) identified ChuA as the major outer membrane transport protein for haemin as a highly conserved oxygenase required for haemn degradation. ChuBCD are most likely involved in the transport of haemin across the inner membrane, although they do not appear essential for this process. A *C. jejuni* chuA mutant was unable to grow on haemin as the sole iron source (Rock *et al.*, 2001). Since the chuB, chuC and chuD mutants were not affected in haemin utilization (Rock *et al.*, 2001), this suggests that the function of the ChuBCD system is complemented by the presence of alternative systems or that *C. jejuni* uses another mechanism for transporting haemn or the haemn iron to the cytoplasm (Henderson *et al.*, 2001; Mourino *et al.*, 2005).

Diverse effects of catecholamines on microorganism have already been observed. Catecholamines have been reported to modulate the growth of gram-negative bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli* (Lyte and Ernst, 1992), and to exert a protective effect against oxidative damage in the pathogenic yeast *Cryptococcus neoformans* (Polacheck *et al.*, 1990). In addition, the ability of some bacteria to utilize these compounds as exogenous siderophore-like molecules in response to iron restriction has been described in some pathogenic bacteria unable to synthesize their own...
siderophores (Yancey and Finkelstein, 1981; Williams et al., 1990; Beall and Sanden, 1995). In Salmonella spp, the effect is thought to be mediated through iron uptake, and involves cell surface siderophore-receptor proteins (Williams et al., 2006). In a recent study, published during the work for this thesis, norepinephrine was shown to enhance the growth of C. jejuni in iron-limiting conditions and also to increase the virulence of this pathogen (Cogan et al., 2007). This study also demonstrates that C. jejuni can acquire iron from DL-norepinephrine and epinephrine, and also from caffeic acid, rutin and quercetin although at high concentrations caffeic acid and rutin were growth inhibitory. C. jejuni was unable to acquire iron from catechin. The role of this in vivo is not known but it may mean that C. jejuni can acquire iron from host hormones or dietary substances.

It has been well documented that exogenously added proline and betaine accumulate as compatible solutes in Gram-positive and Gram-negative bacteria (Dattananda and Gowrishankar, 1989; Townsend and Wilkinson, 1992; Graham and Wilkinson, 1992; Gutierrez et al., 1995). These solutes are often referred to as compatible solutes because they can be accumulated to high levels by de novo synthesis or by transport from the culture medium. Many compatible solutes prove to be effective stabilizers of enzymes, providing protection against high salt (Yancey et al., 1982; Jebbar et al., 1992; Bremer and Krämer, 2000). Compatible solutes are operationally defined as organic osmolytes that can be amassed by the cell in exceedingly high concentrations without disturbing vital cellular functions and the correct folding of proteins (Brown, 1976). Accumulation
of betaine in *Escherichia coli* can be achieved directly by active uptake of betaine or by synthesis from exogenously supplied choline. The osmotically stimulated synthesis of glycine betaine confers high levels of osmotic tolerance to cells grown in media of inhibitory osmolarity (Landfald and Strom, 1986; Styrvold et al., 1986; Dinnbier et al., 1988). Whilst osmoregulation has been extensively studied in other food borne pathogens, the ability of *C. jejuni* to utilize exogenous compatible solutes has not been determined previously. In this work it was observed that exogenous proline and betaine did not stimulate the growth of *C. jejuni* in the presence of high salt concentrations. Proline did stimulate growth but only in the absence of added NaCl. In contrast, glycine betaine appeared to be growth inhibitory irrespective of the osmolarty of the media. Whilst *C. jejuni* might be expected not to use proline or glycine betaine as osmoprotectants given the absence of high affinity transports from the genome sequence (Park, 2005) this is the first time this has been demonstrated empirically.
Chapter 8

General discussion

The aim of present research project was to develop an improved and convenient defined medium for use with Campylobacter species and initially to determine which carbon sources are likely to be used during colonization. The study was extended to determine how the presence of these might influence survival in the environment, to examine possible mechanisms of carbon source utilization, and to the use of the defined medium to study other aspects of Campylobacter physiology.

MEM α was initially assessed as a defined medium but was only found to support growth following the addition of a source of iron, and a buffer to prevent medium acidification. Moreover, high levels of background growth in the absence of added carbon sources may lead to the misidentification of potential carbon sources, and this prevented its further use in this study. Glasgow minimum essential medium (GMEM) (MacPherson and Stoker, 1962; House, 1964) was chosen as an alternative to MEM α because it significantly contains less amino acids and importantly no pyruvate or serine which can be used as carbon sources by C. jejuni. GMEM was also found to be iron-limiting but the addition of 50μM FeSO₄ allowed growth and 25 mM HEPES was added to prevent acidification.
GMEM offered a significant improvement over MEM α because no growth at all was apparent when no additional carbon sources were provided.

The majority of enteric bacteria require a carbohydrate for growth, and utilisation of these is assumed to be the mode of metabolism used by most species (Salyers and Leedle, 1983). In the present study, it was found that L-serine supported *C. jejuni* growth and this has been shown previously to be preferentially utilized compared to other amino acids (Leach *et al.*, 1997). Also L-proline, L-glutamic acid, L-valine, L-glutamine, L-histidine, L-tyrosine, L-asparagine, L- cysteine, and L-glycine all give rise to growth, however, this was modest compared to that seen for L-serine after 30 hours. On the other hand, *C. jejuni* was unable to grow when L- arginine, L-methionine, L-lysine, L- threonine, L- alanine and L- phenylalanine were provided as carbon sources. Amongst the sugars tested in the present study, only fucose, ribose and D-glucosamine hydrochloride supported growth of *C. jejuni* although it was much reduced in comparison to that seen in the presence of pyruvate. Pyruvate has been shown to be the best carbon source to enhance *C. jejuni* growth (Velayudhan and Kelly, 2002).

Most experiments using GMEM were carried out using *C. jejuni* strain NCTC 11168. The general utility of this defined medium, however, was tested by studying the growth of six and five strains of *C. jejuni* and *C. coli* respectively. *C. coli* NCTC 11347, *C. coli* NCTC11366, *C. jejuni* NCTC 11828, *C. coli* NCTC 12110, *C. jejuni* NCTC 11351 and *C. jejuni* NCTC 11168 grew best and achieved increasing levels of growth in this order. These strains grew well in the media suggesting that its use need not only be confined to
the study of the genome strain NCTC 11168. In the case of two of the strains that failed to grow, their growth was stimulated by the addition of the amino acids, either serine or glutamine. This suggests, as noted by Tenover et al., (1985), that certain strains of *Campylobacter* spp. are auxotrophic and GMEM is a broadly applicable defined medium for most *Campylobacter* spp.

Previously it had been shown that mucin preparations enhance the growth of some bacteria (Bradshaw et al., 1994). Taneera et al., (2002) found that porcine gastric mucin stimulated the growth of all five strains of *Helicobacter pylori* tested. *C. jejuni* has also been reported to grow in the presence of mucin alone, although growth curves were not provided in the report (Hugdahl et al., 1988). The present study demonstrated that *C. jejuni* can utilize mucin when it is provided as a sole carbon source since growth in the two defined media was enhanced by its addition. Using a plate based assay for proteineases and mucinases, and using *p*-nitrophenol derivatives of sugars, no proteolytic or glycosidic activities were detected. Either *C. jejuni* does not possess the enzymes or the assays were not able to detect their activities.

Recently, serine transport in *C. jejuni* has been shown to be carried out by a transporter encoded by (SdaC) and the initial stages of its catabolism by a L-serine dehydratase enzyme SdaA (Velayudhan et al., 2004). In the present study, a *sdaC* mutant was constructed to determine if GMEM could be used to analyse the phenotype of the *sdaC* mutant and also to find out whether the inability to use serine affected the ability to utilize mucin. The *sdaC* mutant CJNSA1 was unable to use serine as a carbon source...
because of the lack of activity of SdaC. However, the mutants ability to grow in the presence of mucin was not affected suggesting that mucin does not act as a significant source of available serine.

*C. jejuni* has also been shown to utilize aspartate *in vitro* (Leach *et al.*, 1997). The gene Cj0087 is predicted to encode an aspartate ammonia lyase (AspA) which would liberate oxaloacetate from aspartate. Since oxaloacetate can be fed directly into central metabolism, AspA might be essential for the ability of *C. jejuni* to use aspartate as a carbon source. The present study also found that CJHAR8, an *aspA* mutant, was not able to use aspartate as carbon source. However, the mutant grew well in the presence of mucin and pyruvate. Consequently AspA is obviously necessary for utilization of aspartate by *C. jejuni* but not for growth in the presence of mucin. Therefore, *C. jejuni* is not using mucin as a source of serine or aspartate and consequently must be acquiring other carbon compounds from this molecule.

The capability of *C. jejuni* to use a variety of peptides containing two, four and seven amino acids was assessed since such peptides may be derived from the degradation of mucin. When provided as a sole carbon source, peptides containing two or four aspartate residues induced less growth than a similar concentration of aspartate. Peptides of four and seven amino acids, which also contained serine, stimulated growth in a more dramatic fashion which might indicate that serine in general supports better growth of *C. jejuni* (Chapter 3). The mechanisms via which peptides are transported are not yet known but these must be present in *C. jejuni* as demonstrated by the above experiments.
It was decided therefore to attempt to characterise some of the likely transport mechanisms. In *C. jejuni* genes Cj1580c-1584c are predicted to encode a putative ABC type peptide transporter with homology to Dpp in *E. coli* (Parkhill et al., 2000). Cj1580c is annotated as an ATP-binding protein component of peptide ABC-transport system, while Cj1581c is also annotated as a potential ATP-binding part of a peptide ABC-transport system protein. Cj1582c is annotated as probable peptide ABC-transport system inner membrane permease protein, as is Cj1583c. Cj1584c is annotated as probable peptide ABC-transport system periplasmic peptide-binding protein and has similarity to oligopeptide-binding protein AppA (Parkhill et al., 2000; CampyDB, 2008).

To find out whether Cj1580c-1584c was involved in uptake of peptides, a mutant deficient in this system was acquired from Professor Duncan Maskell’s group at the University of Cambridge. The same group also kindly provided a mutant deficient in Cj0653c, a putative amino peptidase. Since both components were possible candidates for peptide utilization in *C. jejuni* they were assessed, in comparison with the wild type, for growth on the peptides containing serine. Compared to the wild type, growth of neither mutant was diminished, when peptides of four and seven amino acids, also containing serine, were included in the defined growth medium. Thus, Cj1580c-1584c is not involved in the transport of the peptides assessed here but it might be involved in the transport of other peptides with different characteristics. Given the vague specificities of the oligopeptide uptake systems in *H. pylori* (Weinberg and Maier, 2007) this seems probable.
The toxicity of various toxic peptide analogues against *C. jejuni* was assessed as a prelude to using these as selective agents for the isolation of mutants deficient in peptide uptake. Interestingly, *C. jejuni* unlike *Salmonella* and *E. coli* (De Felice *et al*., 1973; Payne, 1983; Morley *et al*., 1983; Higgins and Gibson, 1986) was found to be resistant H-Orn-Orn-Orn-H and Bialophos and this might be due to the different rates of transport of the substrates and to different hydrolysis rates of enzymes within the organism. This is an interesting finding which could lead to the potential use of these two toxic peptide derivatives as selective agents in *C. jejuni* selective media. However, *C. jejuni* was found susceptible to L-Ala-AEP. When different strains of *C. jejuni* and *C. coli* were assessed, for L-Ala-AEP sensitivity, three out of three strains of *C. jejuni* tested were sensitive and three of four stains of *C. coli* tested were resistant. This most likely reflects the existence of different mechanisms of peptide uptake or degradation in strains of *Campylobacter*. Thus resistance in the *C. coli* strains may be due to deficiencies in an uptake system for L Ala-AEP or an alanine aminopeptidase which allows the release of the cell wall inhibitor AEP.

The availability of a defined medium which could easily be rendered iron-deficient by the omission of added FeSO₄ meant that modified GMEM might be suitable for determining the use of iron sources by *C. jejuni*. The present study demonstrated that *C. jejuni* can acquire iron from DL- norepinephrine, epinephrine, caffeic acid, rutin and quercetin but not catechin, although at high concentrations caffeic acid and rutin were growth inhibitors. The role of this *in vivo* is not known but it may mean that *C. jejuni* can
acquire iron from host hormones or dietary substances (Yancey and Finkelstein, 1981; Williams et al., 1990; Beall and Sanden, 1995; Cogan et al., 2007).

The capacity of *C. jejuni* to utilize exogenous compatible solutes during growth at elevated osmolarities has not been determined previously; the availability of the defined media provided an opportunity to study this. In the present work, it was observed that exogenous proline and betaine did not stimulate the growth of *C. jejuni* in the presence of high salt concentrations. Proline did stimulate growth but only in the absence of added NaCl (Chapter 7) and most likely because it can be used as a carbon source. In contrast, glycine betaine appeared to be growth inhibitory irrespective of the osmolarity of the media. Whilst *C. jejuni* might be expected not to use proline or glycine betaine as osmoprotectants given the absence of high affinity transports from the genome sequence (Park, 2005) this is the first time this has been demonstrated empirically.

In the initial stages of the present study a number of carbon sources, that could be used by *C. jejuni* had been identified. Whilst the survival of *C. jejuni* has been studied extensively (Rollins and Colwell, 1986; Cools et al., 2003; Richardson et al., 2007), little is known about how the availability of carbon sources affects its survival. Consequently the effect of carbon source on survival was studied. Mucin was clearly protective and cells survived better in its continual presence. Moreover, when it was added to cells that had survived in its absence, recovery of viable cells was increased. This effect was related to the concentration of mucin with 0.1% having no effect and 1%, the highest concentration used, stimulating and increase in cell count of at least 10-fold. It is possible
that by attaching to mucin in the medium, *C. jejuni* may form cell clumps and as this might protect cells from the harmful effects of the oxygen dissolved in the surrounding liquid (Jiang and Doyle, 2000) it might account for the enhanced viability observed. However, the formation of clumps would initially result in an apparent decline in cell number, when actually there was an increase. This mechanism is thus unlikely to explain the protective effect of mucin. It is possible that the increased viscosity provided by mucin in the supplemented MRD may have also created a more favourable microaerobic environment for *C. jejuni* survival as this would inhibit oxygen diffusion (Cellini *et al.*, 1994; Federighi *et al.*, 1998).

When carbon sources were added as single and defined compounds the effect on viability was negative to that observed for mucin, in that the addition of pyruvate, serine and aspartate brought about a rapid decline in viability. This phenomenon clearly required that the additive be a usable carbon source since when threonine, which cannot be used as a sole carbon source, was added no decrease in viability was measured. In balanced medium (nutrient broth), the addition of pyruvate results in a decrease in the concentration of hydrogen peroxide, which enables *C. jejuni* to grow aerobically (Verhoeff-Bakkenes *et al.*, 2008). In the present study the mostly likely explanation for the negative effect on viability of pyruvate and other single carbon sources, may be by the "substrate accelerated death" phenomenon whereby death ensues due to over accumulation of carbon sources (Postgate and Hunter, 1963; Dykhuizen and Hartllactose, 1978; Postma *et al.*, 1990).
8.1 Future work

The research presented here raises a number of interesting avenues for further research. The uptake of amino acids and fucose could be further characterised by identifying, and mutating the transporters responsible. The genome encodes for example, putative transporters for fucose (Cj0486) and proline (Cj1502c). Campylobacters are able to utilise mucin as a carbon source but the mechanisms of mucin utilization have not been established. Whether or not these pathogens have mucinolytic activity could be verified by exposing mucin to \textit{C. jejuni} and assaying for breakdown products using polyacrylamide electrophoresis or mass spectrometry. \textit{C. jejuni} is able to use peptides as carbon source and an effective screen for isolating mutants deficient in peptide transport, and degradation mechanisms was developed based on L-Ala-AEP. It should prove possible, using an improved and more complete transposon library to isolate and characterise mutants deficient in these systems. One of the most interesting areas identified in this study is the effect of carbon sources on the recovery of \textit{C. jejuni} during survival. It is clear that mucin enhances recovery when added to surviving cells. Using other measures of viability such as the LIVE/DEAD BacLight kit (Chaiyaan \textit{et al.}, 2001; Grey and Steck, 2001; Mary \textit{et al.}, 2002). This kit contains two nucleic acid stains which distinguishes viable bacterial cells from dead ones on the basis of membrane integrity. SYTO 9 (which gives green fluorescence) can pass through the intact plasma membrane of bacterial cells due to its low molecular weight, while the larger red fluorochrome (propidium iodide) penetrates only compromised membranes. Bacterial suspensions incubated in the presence of both stains simultaneously will fluoresce either green (i.e.,
viable) or red (i.e., dead), depending on their viability (Alonso et al., 2002; Sachidanandham et al., 2005). It might be possible to study this effect further. In addition, it would be interesting to fractionate the mucin and determine whether there are specific components in it that were responsible for the stimulation of viability. In this study substrate-induced death was identified for the first time in C. jejuni but the mechanism has not been established. This could be further investigated by using mutants sensitive to oxidative stress (sodB, katA) to determine whether it is induced by oxidative stress. In addition, intracellular levels of the carbon sources responsible could be monitored during substrate accelerated death to determine whether it results from over accumulation of the carbon sources.
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INTRODUCTION

- *C. jejuni* generally known to grow at the expense of amino acids in vitro and has been shown to utilize serine, aspartate, glutamate, and proline (Leach et al., 1997). L-serine is preferentially utilized compared to other amino acids and catabolism of L-serine to pyruvate and ammonia is carried out by a series dehydratase (encoded by Cj1624c) (Velayudhan et al., 2004).

- Campylobacter colonizes the intestinal mucus layer in the crypts of the intestinal epithelium. Mucus which is continually secreted by intestinal epithelial cell is rich in nutrients and can also readily support the growth of Campylobacter (Beery et al., 1988).

**Materials and Methods**

Liquid cultures 50 ml of *C. jejuni* were grown microaerobically with gentle agitation in different media with supplemented FeSO4 50 µM and HEPES 25mM when necessary in tissue culture flasks with vents. Two defined media were investigated. Minimum Essential Medium (MEM, Invitrogen) and Glasgow Minimum Essential Medium liquid (G-MEM, Invitrogen). G-MEM was used as supplied whilst MEM was made up according to the manufacturers instructions. The growth of *C. jejuni* was studied in GMEM and MEM with mucin, sodium pyruvate, various amino acids and sugar provided as potential carbon sources.

**Results**

Selection of the suitable defined media for growth of *C. jejuni*

This involved assessment of ability of *C. jejuni* to use different compound as sole carbon sources (Fig. 1). The defined media tested were MEM and GMEM. Initially it was chosen to assess the ability of *C. jejuni* to use mucin, amino acid and sugar as a sole carbon sources. Growth was only apparent in MEM a following the addition of mucin. Consequently, this media is considered unsuitable for detailed analysis of carbon source utilization by *C. jejuni*.

**Conclusions**

The conclusions has shown that GMEM is amoue suitable defined media and provided novel data on utilization of carbon sources by this organism.

**References**


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